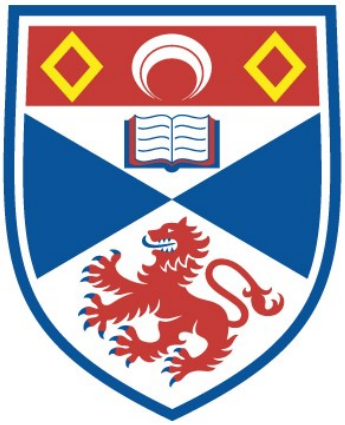
STUDIES ON TWO CONSTITUENTS OF ELASTIC TISSUE: ELASTIN AND THE MICROFIBRILLAR COMPONENT

John Michael Field

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1975

Studies on Two Constituents of Elastic Tissue: Elastin and the Microfibrillar Component.

A Thesis presented by

JOHN MICHAEL FIELD

to

The University of St. Andrews

in application for the

Degree of Doctor of Philosophy

1975

Studies on Two Constituents of Elastic Tissue: Elastin and the Microfibrillar Component

A thesis presented by J.M. Field to the
University of St. Andrews in application
for the Degree of Doctor of Philosophy.

Abstract

Section A: Insoluble elastin was isolated from bovine aorta and ligamentum nuchae by the use of guanidine and dithiothreitol in conjunction with collagenase, purified by affinity chromatography. The preparations were free from carbohydrate and exhibited amino acid compositions similar to that of alkali-purified elastin, with the exception of the concentration of some polar amino acid residues. N-terminal analyses indicated a very low level of polypeptide chain damage in the preparations. Upon mild alkaline treatment glycine was selectively liberated as amino-end group.

Elastin from ligamentum nuchae was examined by electron microscopy, X-ray diffraction, optical polarisation analysis and dynamometry. It is suggested that elastin fibrils consist of a lateral array of primary filaments.

Section B: The microfibrillar component of adult bovine ligamentum nuchae was isolated by solubilisation in guanidine-dithiothreitol followed by S-carboxymethylation and treatment with collagenase. The amino acid composition of the material was at variance to those previously reported for similar preparations. N-terminal analysis revealed glycine as the only end-group, at a concentration of 65.9 moles/10^6 g of protein. This value, corresponding to a molecular weight of about 15,000 daltons, was in good agreement with the results of sedimentation equilibrium analyses.

CERTIFICATE.

I herby certify that J.M. Field has spent nine terms engaged in research under my direction and that he has fulfilled the conditions of Ordinance General No.12, and Resolution of the University Court 1967, No.1, and that he is qualified to submit the accompanying Thesis for the Degree of Doctor of Philosophy.

DECLARATION

I hereby declare that the following thesis is based on work performed by me, that the thesis is my own composition and that no part of it has been presented previously for a higher degree.

The research was conducted in the Department of Biochemistry, United College of St. Salvator and St. Leonard, University of St. Andrews, under the direction of Dr. A. Serafini-Fracassini.

ACADEMIC RECORD

I first matriculated at the University of St. Andrews in October, 1968, and graduated with the Degree of Bachelor of Science, Upper Second Class Honours in Biochemistry, in June 1972. I matriculated as a research student in the Department of Biochemistry, University of St. Andrews in October, 1972.

ACKNOWLEDGEMENTS.

I should like to express my gratitude to Dr. A. Serafini-Fracassini for his help and guidance during the course of this work. My thanks are also due to Mr. J.C. Hunter for the operation of the amino acid analysers, to Mr. C. Armitt for the operation of the ultracentrifuge, to Mr. W. Blyth for photographic work, and to Dr. B. Delf for X-ray analyses.

This work was supported by a grant from the Scottish Home and Health Department.

CONTENTS

SECTION A

ELASTIN

INTRODUCTION

Elastin is a widespread structural protein which exhibits some remarkable physical properties, the most obvious and striking of which is its ability to sustain large deformations without rupture and to revert spontaneously to its original condition when tension is released.

Elastin occurs together with collagen in almost all animal connective tissues, but in most of these it is present only as a minor component. When there is a need for elastic extension and recovery, as, for instance in the wall of the large arteries or the ligament which supports the head of ruminants, elastin comprises the greater part of the structure, up to 60 or 70% of the dry-weight of the tissue (1). Elastin has also been identified, both histologically and chemically, as a prominent component of the intercellular matrix in a type of cartilage which is restricted in its anatomical location mainly to the epiglottis, external ear and cartilagenous pharangeal-tympanic tube. However, since the isolation of the protein from cartilagenous sources presents special difficulties, most biochemical investigations have been made on elastin from the tunica media of large arteries and the ligamentum nuchae of large ruminants, from which the protein is more easily isolated.

Table 1, below, reports the content of elastin in various tissues.

Table 1 (1)/

Table 1 (1)

Tissue	Elastin content g/100g dry tissue
Aorta	51.0
Ligamentum nuchae	70.0
Lung parenchyma	12.5
Pleura — Parietal	14.7
Pleura — Visceral	27.8
Trachea	0.9
Major Bronchii	1.4

Elastic tissue occurs in two main structural forms depending upon its anatomical location. In ligament, skin and loose connective tissue it is present as fibres while in the walls of blood vessels it exists in the form of lamellae. Both fibres and lamellae are constituted by fibrils which, as revealed by electron microscopy, are embedded in an amorphous matrix in close association with collagen (2). It is this intimate relationship between elastin and other macromolecules that renders the isolation of the protein so difficult, particularly as both collagen and elastin are insoluble in those solvents which are normally employed for the extraction of tissue proteins.

Mature

Mature elastin exhibits its characteristic high degree of insolubility by virtue of its high content of covalent cross-links. The structures of three of these cross-links, namely desmosine, isodesmosine and lysinonorleucine are shown in Figure 1. Desmosine and isodesmosine are designated, respectively, as 1,3,4,5, and 1,2,3,5, - tetra substituted pyridinium salts (3,4). They exhibit a characteristic ultraviolet absorption spectrum with a maximum at 275 nm (4). The chain at position 1 on the pyridinium ring contains six carbon atoms in both isomers (4,5) while the length of the other three side chains depends upon the position of substitution. Examination of the structure of desmosine and isodesmosine shows them to be potentially capable of uniting up to four polypeptide chains, as each side chain carries both a carboxyl and an α- amino group. It would appear that the degree of cross-linking of elastin may be significantly different in various tissues, particularly in auricular cartilage elastin, as the quantitative determination of desmosine and isodesmosine in elastin hydrolysates has revealed that the concentration and molar ratio of the two polyfunctional amino acids depends upon the source of the elastin preparation (6,7).

The third common polyfunctional amino acid isolated from elastin hydrolysates is lysinonorleucine, first identified in elastin from bovine ligamentum nuchae (8). The structure of this compound, N^{ϵ}- (5- amino - 5 - carboxypentyl) lysine (9), suggests

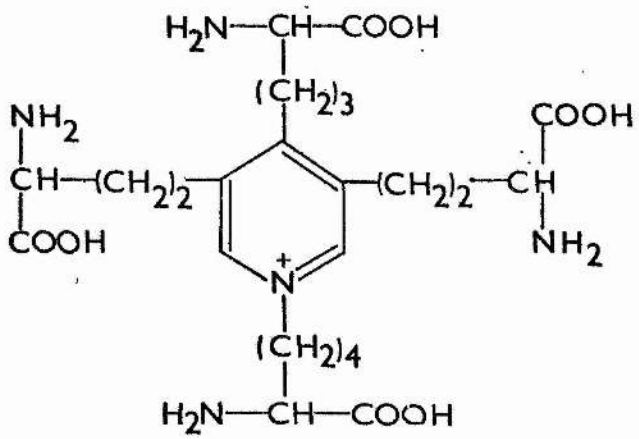
that/

Fig. 1. The structures of Desmosine, Isodesmosine and Lysinonorleucine.

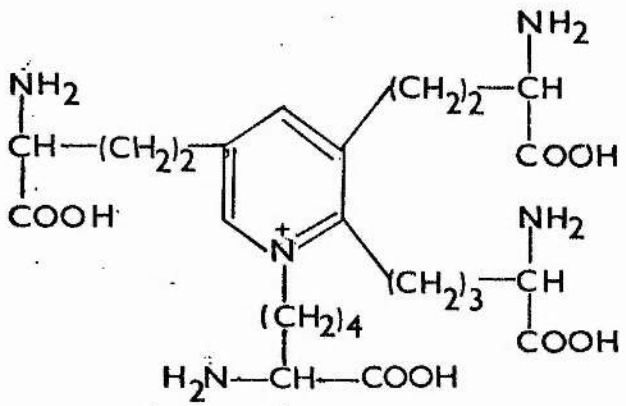
a : Desmosine

b : Isodesmosine

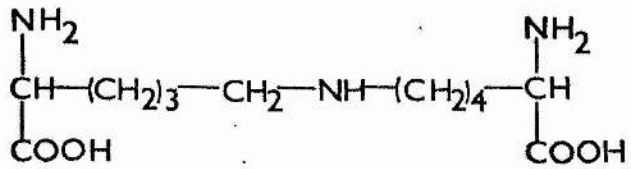
c : Lysinonorleucine



a



b



c

that one such residue would unite two polypeptide chains.

Although the desmosines and lysinonorleucine are the most common cross-links in elastin several other cross-links or cross-link precursors are present in the protein (10,11,12).

The presence of polyfunctional amino acids covalently cross-linking polypeptide chains in elastin prevents any solubilisation of the protein without the prior cleavage of either specific peptide bonds of the protein backbone or carbon-carbon bonds of the pyridinium rings of desmosine and isodesmosine. This partial fragmentation of the protein and its subsequent solubilisation may be brought about by the use of enzymes, such as pancreatic elastase (EC 3.4.4.7) or papain (EC 3.4.4.10), or by non-enzymic cleavage with a reagent such as 0.25 M oxalic acid at 100 °C (13-16). Treatment of elastin with oxalic acid results in the complete solubilisation of the protein. The soluble material may be separated intotwo fractions, designated α- and β - elastin. Several structural investigations have been performed on α - elastin, but the extrapolation of conclusions derived from these studies to include insoluble elastin must be undertaken with caution.

Because of the complete insolubility of intact mature elastin, the isolation of the protein from connective tissue matrices must rely upon the removal of all other tissue components. The most frequently adopted procedures for the purification of elastin involve either the use of hot alkali (17), prolonged thermal treatment (15) or a combination of the two. Elastin, at variance with

collagen,/

collagen, shows considerable stability to these treatments in that it is not solubilised to any great extent. However, the identification of a large number of N - terminal amino acid residues in elastin preparations isolated by the use of these procedures (Table 2) (18) clearly indicates that such extremes of temperature and pH catalyse the hydrolysis of several peptide bonds. This may lead to the loss of labile segments of the elastin polypeptide chain and affect the mechanical properties of the protein. As most chemical and physico-chemical studies have been carried out on elastin preparations isolated by these techniques it would perhaps be desirable to repeat the investigations upon samples of the pure protein which have not been subjected to such harsh conditions during purification.

In an attempt to minimise chain cleavage and to isolate elastin in a conformation approaching more closely that occurring in the native state, these procedures are being replaced by others in which contaminating structural components of the tissue are selectively extracted, or hydrolysed by specific enzymes (19-23). The main problem in this type of procedure is to achieve complete removal of all unwanted components without causing damage to the elastin, since the possibility exists that the enzyme or enzymes used may possess some degree of activity towards this protein. Although the high specificity of a particular enzyme may preclude it from digesting elastin, its commercially available preparation may be contaminated by elastases or non-specific proteases. In this

respect/

Table 2. (18)

N - terminal amino acid residues of elastin from bovine ligamentum nuchae (values are expressed as moles of amino acid per 10^6g of protein. Results are corrected for regeneration losses).

Method of Purification	Thermal Treatment	Thermal treatment followed by Alkaline Treatment
Aspartic Acid	0.34	0.19
Threonine	0.06	0.12
Serine	0.27	0.25
Glutamic acid	0.05	tr.
Proline	0.20	0.94
Glycine	1.28	8.99
Alanine	0.72	2.80
Valine	0.23	0.96
Isoleucine	0.10	0.18
Leucine	0.34	0.59
Tyrosine	0.06	0.11
Phenylalanine	0.07	0.27
Totals	3.72	15.40

respect it should be mentioned that α- amylase (EC 3.2.1.1) preparations used to remove mucoprotein from collagen so as to allow dispersion of collagen fibrils in dilute acetic acid (21), exhibit proteolytic activity, possibly as a result of subtilisin (EC 3.4.4.16) contamination (24), while some collagenase (EC 3.4.4.19) preparations have pronounced elastolytic activity (25). Before proceeding with an enzymic purification of elastin it is essential that an assay be made of possible elastolytic activity in the enzyme preparation, which may require further purification.

It is not at present known whether elastin is identical in all tissues or if, in fact, there exists a family of similar proteins which show a tissue specificity. Confusion has arisen as a result of the considerable variation in the ease with which the elastic fibres in different sites may be purified by chemical means. Certainly the amino acid compositions of elastin preparations vary according to the species and tissue under investigation and the age of the animal but they also vary according to the isolation procedure. Whether there are in fact true species -, tissue- and age-related differences in the composition of the protein or that these differences have arisen as a consequence of the variation in the ease of removal of contaminating proteins it is impossible to say (6,26-35).

Much interest has been shown in recent years in a soluble protein which was first isolated by salt-extraction from the aorta

of/

of copper deficient calves (36). This protein, which like α - elastin was susceptible to reversible heat precipitation, was found to possess an amino acid composition very similar to that of insoluble elastin with the exception that it was lacking in desmosines and was comparatively richer in lysine (37). A very similar preparation was obtained by Sandberg and his colleagues upon extraction of aortic tissue with formic acid, although this acid-soluble protein showed a greater degree of polydispersity than the salt-extracted preparation (38).

Labelling experiments carried out with (^{14}C) valine very strongly indicated that the small amount of soluble protein, isolated from normal newborn pig aorta and apparently identical to the salt-soluble preparation from copper deficient animals, was a rapidly cross-linked precursor of elastin (39). In copper deficient or lathrytic animals the oxidation of lysyl residues by lysyl oxidase which normally occurs in the synthesis of cross-links is inhibited (40). It is now accepted that this soluble protein, present usually only at low concentrations, is the physiological precursor of insoluble cross-linked elastin. The molecular weight of tropoelastin, as the soluble protein was named, varies according to the isolation procedure, between 30 000 and 100 000 daltons (41,42); a recent investigation in which proteolysis was eliminated indicated the molecular weight to be 74 000 daltons (43).

Tropoelastin/

Tropoelastin is characterised by the presence of multiple repeats of the sequences, Lys - Ala - Ala - Ala - Lys and Lys - Ala - Ala - Lys (41,42,44). It is considered that these sequences are the regions of the chain which are involved in the formation of the cross-linkages typical of the insoluble protein (41). Earlier work on the amino acid composition of the cross-link regions showed that desmosine - rich peptides from insoluble elastin also exhibited a high content of alanine (3,45-47). It has been suggested that elastin polypeptide chains may be constituted of long sequences of apolar amino acid residues separated by polar regions rich in cross-links. (48)

For many years the criterion for the assessment of the purity of new preparations of elastin has been the comparison of their amino acid profiles with that of elastin purified by treatment with hot alkali (17). However, the identification of a large number of amino end-groups (18) in hot alkali purified elastin clearly indicates that the treatment induces the hydrolysis of several peptide bonds and the possible release of segments of the protein polypeptide chain, as previously stated. It might be expected that the peptide bonds most labile to cleavage by sodium hydroxide would be those in the vicinity of polar amino acid residues. In this respect it is significant that enzymically purified insoluble elastin exhibits concentrations of acidic and hydroxy amino acids substantially higher than those reported for

sodium/

sodium hydroxide treated preparations (1).

It would appear, therefore, that a more reliable indication as to the degree of purity of insoluble elastin preparations might be obtained if comparison was made with the amino acid composition of tropoelastin, with due allowance for the utilisation of lysyl residues in cross-links.

The ultrastructural arrangement of elastin is still a matter for conjecture. In conventionally stained sections of fixed tissue elastin fibrils present an amorphous appearance. However, the amorphous nature of the protein has been questioned on the grounds that elastin, isolated by either thermal (15) or alkaline treatment (17) can be resolved into longitudinal filamentous units (49,31) when subjected to ultrasonic dispersion for a prolonged period of time and examined by negative contrast. These filaments, which have a diameter of less than 3 nm (50), are seen to run parallel to the major axis of the fibril and show a 'beaded' appearance. However, the interpretation of this negative staining pattern as indicating that the elastin fibril consits of closely packed filaments has not found full acceptance. Strong reservations have, in fact been expressed concerning the efficiency of the purification procedures in removing contaminating materials, primarily collagen (51), and the effect of sonication on the conformation of the protein (15). In addition such ultrastructural organisation of elastin has failed to find support from both x-ray

and/

and circular dichroism analyses conducted on elastin and α-elastin (13), as neither technique revealed the presence, in general, of any definite secondary or tertiary structure (52,53).

As initially remarked, the most striking property exhibited by elastin is that of elasticity, viz. elastin responds to an external force by a large but temporary deformation without rupture. The stress-strain curves of both untreated ligamentum nuchae and purified elastin are remarkably similar up to elongations in the order of 70%, both materials obeying Hooke's law and exhibiting a Young's modulus of about 10^7 dynes cm^{-2}, providing the samples have a water content greater than 40% of the total sample weight (52). This value for the Young's modulus is within the range of values reported for typical rubbers. Above elongations of about 70% purified elastin specimens undergo rupture, while those of untreated ligamentum nuchae show an abrupt increase in modulus (52). Elastic elongations of 70% at yield obtained with gross samples of purified elastin, are very low in comparison to elongations in the range 300 - 400% recorded with vulcanized rubber.

In a perfect rubber, elasticity is a consequence of the presence of kinetically-free chains which are cross-linked so as to form a three-dimensional network, a structure in which chain slippage is prevented. The heat evolved on stretching such a system is approximately equivalent to the work required to perform the extension (54), the driving force for contraction being purely

entropic./

entropic. In the case of elastin, the heat produced during stretching, in water, is greater than that corresponding to the work performed in elongating the sample(55). Furthermore, it was found that this excessive release of heat was minimised by increasing the concentration of organic solvents in the swelling medium (55). This observation lead Weis-Fogh and Andersen (56) to propose a novel model for elastin elasticity. In this model, which presupposes that large segments of the polypeptide chains are coiled into globular structures in relaxed specimens, increasing numbers of apolar side chains are brought into contact with solvent upon extension of the sample and deformation of the microscopic globular units. The driving force for recoil would then be provided by the tendency of the protein to re-acquire the conformation in which the interaction of water and hydrophobic side chains is minimised. This is brought about by contraction of the polypeptide chains so as to bury apolar residues in the interior of the hypothetical globular structures. A model of this nature is certainly compatible with the observation of 'beaded' filaments in elastin specimens (50).

In a recent publication (57) Hoeve and Flory have demonstrated that the Weis-Fogh and Andersen model of elastin is incapable of quantitatively accounting for the energetics of elastin elasticity, and have successfully explained the anomalous thermodynamic behaviour of elastin on the basis of solvent dilution in an other-

wise/

wise rubber-like system. The authors state that the macromolecular organisation of elastin would, therefore, appear to conform to that of rubber, i.e. a system of kinetically-free chains forming a three dimensional network stabilised by cross-linkages.

Section A of this thesis describes the isolation of insoluble elastin by a mild enzymic procedure and the examination of some of the chemical and physical properties of the preparation.

Elastin was isolated from bovine ligamentum nuchae and aorta by a slightly modified version of the procedure of Ross and Bornstein (22). The tissues were serially extracted with 5M guanidine and dithiothreitol in 5M guanidine, followed by digestion with collagenase (EC 3.4.4.19), purified either by gel exclusion and ion-exchange chromatography (58) or by affinity chromatography following batchwise fractionation on diethylaminoethyl cellulose. The collagenase preparations were assayed for elastolytic activity by estimation of the soluble peptides released from the substrate, namely insoluble collagen-free elastin, prepared from bovine ligamentum nuchae by hot alkali treatment (17). The chemical integrity of the elastin preparations isolated in this way was assessed by estimation of their N-terminal profiles. It was felt that this would give a more accurate indication of the presence of elastolytic activity in the enzyme preparation than analyses carried out on the digestion supernatant as described above, since owing to the highly cross-linked nature of elastin, substantial

peptide/

peptide bond cleavage may occur without liberation of peptide fragments.

Enzymically purified elastin preparations were characterised with respect to their amino acid and carbohydrate composition. As none of the N-terminal residues identified in the elastin preparations was present in sufficient amount to be compatible with the molecular weight of tropoelastin (74 000 daltons)(43) attempts were made to discover the nature of the true end-group of the protein.

Previous studies (52,59) of the stress-strain relationship in elastin were conducted upon samples purified by either thermal or hot-alkali treatment (15,17), procedures shown to impair the integrity of the protein (18). It was decided to re-examine the mechanical properties of elastin using undegraded, enzymically purified specimens in order to determine whether the reported behaviour (52,59) of elastin was typical of the protein or had, in fact, been modified as a result of peptide bond cleavage during the purification procedures.

A re-investigation was also made of the electron microscopic appearance of elastin. Finely powdered specimens of an enzymically purified preparation were examined by negative contrast. To eliminate the possibility of conformational changes induced by sonication (3), the routine use of this treatment was avoided.

Dimethyl/

Dimethyl sulphoxide, known to swell elastin (59,60), was occassionally used to facilitate the diffusion of stain into the interior of the fibrils.

The structural arrangement of enzymically purified elastin was also investigated by both optical polarisation and X-ray diffraction analysis in an attempt to gain additional data to aid in the interpretation of electronmicroscopic evidence.

MATERIALS AND METHODS

Purification of Clostridium histolyticum collagenase (clostridiopeptidase A, EC 3.4.4.19)

a). Gel - and Ion Exchange - Chromatography

An aliquot (400 mg) of a commercial collagenase preparation (Type 1, Sigma (London) Chemical Co., Ltd.), dissolved in 30 ml 5 mM Tris (pH 7.5) containing 4mM $CaCl_2$, was applied to a column (5.0 x 100 cm) of Sephadex G200 (Pharmacia Fine Chemicals, Sweden) equilibrated with the same buffer. Elution was performed at 4 °C with a flow rate of 22 ml/h, fractions of 6 ml being collected and monitored for protein content by ultraviolet spectrophotometry at a wavelength of 275 nm. The collagenolytic and non-specific proteolytic activity of every fifth fraction was also monitored, as will later be described.

Those fractions, from three such chromatographic runs, containing collagenolytic activity were pooled (giving approximately 400 ml of solution) and applied to a column (2 x 10 cm) of diethylaminoethyl (DEAE) cellulose (DE 52 cellulose, Whatman Biochemicals Ltd.) equilibrated with 5mM Tris (pH 7.5) containing 4 mM $CaCl_2$. The column was then washed, at 4 °C, with the same buffer at a flow rate of 40 ml/h until protein could no longer be detected in the effluent by ultraviolet spectrophotometry at 275 nm. The eluant was changed to 50 mM Tris (pH 7.5) containing 4 mM $CaCl_2$. When the effluent was again free of protein the eluant was changed to 5mM Tris (pH 7.5) containing 4 mM $CaCl_2$ and

100 mM/

100 mM NaCl, and finally to 5mM Tris (pH 7.5) containing 4 mM $CaCl_2$ and 500 mM NaCl (58). The experimental conditions were identical for each buffer solution, that is a flow rate of 40 ml/h at 4 °C with 6 ml fractions being collected and stored at 4°C. The protein content, as indicated by the absorbance at 275 nm, of each fraction and the collagenolytic and non-specific proteolytic activity of every fifth fraction were monitored. Suitable fractions were pooled, dialysed against 0.1 mM $CaCl_2$ (collagenase being much more stable in the presence of calcium ions than in pure water) at 4°C and lyophilised.

Before use in the purification of elastin aliquots of the lyophilised material were tested for the presence of elastolytic activity using insoluble elastin, isolated from bovine ligamentum nuchae by hot alkali treatment (17), as a substrate.

b). Affinity Chromatography

Before a purification by affinity chromatography was attempted much of the brown pigment and non-specific proteolytic activity associated with the crude collagenase preparation (Type 1, Sigma (London) Chemical Co., Ltd.) was removed by treatment with DEAE cellulose (De 52 cellulose, Whatman Biochemicals Ltd). An aliquot of the crude enzyme preparation, dissolved in 30 ml of 5 mM Tris (pH 7.5) containing 4 mM $CaCl_2$ and 100 mM NaCl, was added to 20g of pre-swollen DEAE cellulose suspended in 20 mls of the same buffer and stirred for 1 hr at 4 °C. The ion-exchanger was then removed by low speed centrifugation for 15 minutes at 4 °C, the

supernatant/

supernatant added to a fresh suspension of DEAE cellulose and the process repeated. The resulting solution of partly purified collagenase was dialysed exhaustively against 0.1 mM $CaCl_2$ at 4 °C and lyophilised. Lyophilised material was stored at -40 °C.

Although the technique of affinity chromatography can yield highly purified preparations it can suffer from the effects of the non-specific adsorption of contaminants. These contaminants are often removed from the affinity support under the same experimental conditions as those necessary to effect the removal of the required material resulting in a final preparation which, though much purified, contains undesirable components. In order to circumvent this problem it was decided to utilise a support with affinity for those proteins in the collagenase preparation possessing elastolytic activity, rather than a support which would retain collagenase. An obvious choice for such a support was that of insoluble elastin from which any collagen had been removed. Coarsely milled bovine ligamentum nuchae was subjected to the hot alkali treatment of Lansing et al (17) to yield collagen-free elastin. An aliquot of this material was swollen in 15 mM Tris (pH 7.5) containing 1 mM $CaCl_2$, degassed under reduced pressure and used to pack a small column (1.3 cm x 6.0 cm) which was washed with 15 mM Tris (pH 7.5) containing 1 mM $CaCl_2$ until no material could be detected in the effluent by scanning ultraviolet spectrophotometry. To this column was applied, under gravity, a solution of the partly purified collagenase preparation

(20 mg/

(20 mg in 5 ml 15 mM Tris (pH 7.5) containing 1 mM $CaCl_2$. The column was eluted at 4 °C with the Tris - $CaCl_2$ buffer at a flow rate of 20 ml/h. The column effluent was monitored by scanning ultraviolet spectrophotometry and the first 18 ml collected and stored at 4 °C. The enzyme preparation was found to retain its activity for several weeks when stored under these conditions. Before use in the purification of elastin all collagenase solutions prepared in this way were checked for elastolytic activity.

Estimation of Collagenolytic Activity

Aliquots (0.5 ml) of enzyme solution, containing a suitable amount of enzyme, were added to 9.5 ml of 0.1M Tris (pH 7.5) containing 10 mM $CaCl_2$ and 10 mg of insoluble collagen (isolated from calf skin). The mixture was incubated at 37 °C for 2 hrs. after which time it was filtered. In the case of the control 0.5 ml of enzyme solution was added to a collagen suspension which had undergone a 2 hr incubation at 37 °C. The suspension was filtered immediately after the addition of the enzyme.

The amount of soluble material which had been released by the action of collagenase was then estimated using ninhydrin to detect free amino groups (61). To 0.5 ml of filtrate was added

2 ml/

2 ml ninhydrin solution (1.2% solution of ninhydrin in methoxy-ethanol - sodium acetate (pH 5.5) containing 0.033% stannous chloride as antoxidant) and the solutions mixed thoroughly for 2 minutes using a Vortex mixer. After 15 minutes at 100 °C the reaction mixture was cooled, diluted with 10 ml of 50% (volume by volume) aqueous n-propanol and the absorbance of the solution at 578 nm read after 10 minutes at room temperature.

A solution of leucine of known concentration was used to prepare a calibration curve.

Estimation of Non-Specific Proteolytic Activity

To 4 ml of a 0.6% solution of casein in 0.1M Tris (pH 7.8) was added 0.1 ml enzyme solution. After a 1 hr. incubation at 37°C 2 ml of 25% (weight by volume) trichloracetic acid sdution were added, and the mixture incubated for a further 30 minutes at 37 °C prior to filtration. The concentration of peptides, too small to be precipitated by trichloracetic acid, released from the casein by the activity of non-specific proteases was then estimated on a 0.5 ml aliquot of the filtrate using ninhydrin as described for the assay of collagenolytic activity. A control was prepared by the addition of the enzyme solution after the addition of trichloracetic acid to the casein solution.

Estimation/

Estimation of Elastolytic Activity

Aliquots (0.5 ml) of enzyme solution were added to 9.5 ml of 0.1M Tris (pH 7.8). containing 10 mg of finely powdered, insoluble collagen - free elastin (purified by treatment with hot alkali). The suspension was incubated at 37°C for 24 or 48 hrs. after which insoluble material was removed by filtration. The amount of protein, solubilised by the action of enzymes possessing elastolytic activity, present in 0.5 ml of filtrate was estimated using ninhydrin as already described. The control consisted of 10 mg of elastin in 9.5 ml 0.1M Tris (pH 7.8) which was incubated for either 24 or 48 hrs. at 37°C before the addition of 0.5 ml enzyme solution. The suspension was filtered immediately following the addition of enzyme.

Because of the cross-linked nature of insoluble elastin, substantial peptide bond damage might occur without the accompanying release of peptides. As any damage of this nature would remain undetected by analyses performed upon the digestion filtrate it was felt that a more reliable estimate of the amount of damage caused by elastolytic enzymes would be provided by the determination of amino-end groups present in the insoluble elastin purified by the use of collagenase preparations.

The Isolation of Insoluble Elastin from Bovine Ligamentum nuchae

a). Collagenase in conjunction with Guanidine Hydrochloride and Dithiothreitol

A modified version of the procedure originally described by

Ross/

Ross and Bornstein (22) was adopted for the isolation of insoluble elastin from adult bovine ligamentum nuchae.

The ligamentum nuchae of three-year old cattle was homogenised in ice cold water and then dehydrated and defatted in chloroform-methanol (3:1 by volume) for 24 hrs. at 4°C. The defatted material was collected by centrifugation, air-dried and finely ground in a hammer-mill (Stanmore Glencreston). The resulting cream-coloured powder was graded on sieves of the ASTM series and the 100 - 200 mesh fraction (149-79 micron particle diameter) extracted with 1% NaCl for 24 hrs at 4°C, in the presence of a small amount of toluene as a bacteriostatic agent. The residue was collected by centrifugation and washed thoroughly with water prior to lyophilisation.

An aliquot (1g) of the defatted, saline washed preparation was extracted for six 24 hr. periods with 5 M guanidine hydrochloride (Sigma (London) Chemical Co., Ltd., 'practical grade' which was recrystallised before use from both ethanol-benzene and water according to the method of Nozaki (62)) in 0.1 M Tris (pH 7.4) at 4°C. In order to ensure that the maximum quantity of guanidine soluble material was removed, the levels in the extraction supernatant of both hexosamine and hydroxyproline were estimated after each 24 hr extraction period.

The extraction residue was next treated with dithiothreitol in order to solubilise those proteins possessing inter-molecular

disulphide/

disulphide bonds. The preparation was suspended in 5M guanidine hydrochloride in 0.1M Tris (pH 7.4) containing 0.4% ethylene-diamine tetracetic acid (disodium salt, EDTA). Prior to the addition of dithiothreitol (Sigma (London) Chemical Co., Ltd.) to a concentration of 0.05M the suspension was degassed under reduced pressure and then placed under a nitrogen barrier. The extraction was allowed to proceed with stirring at 37 °C for 24 hrs after which time the residue was collected by centrifugation and resuspended in fresh extraction medium for a further 24 hr. period. The level of hexosamine and hydroxyproline in the extracts was again estimated. The extraction residue waswashed with 5M guanidine hydrochloride in 0.1M Tris (pH 7.4) followed by water and lyophilised.

Finally this material was treated with collagenase; the residue was suspended in 0.01M $CaCl_2$ (containing a small quantity of toluene) and the pH of the suspension adjusted with 0.01N NaOH to a value of 7.5. When the residue had fully equilibrated and the pH of the suspension showed no tendency to drift, purified collagenase was added to give an enzyme to residue ratio of approximately 1: 1000. The digestion was allowed to proceed at 37 °C and pH 7.5, this value being maintained - and the rate of carboxyl group liberation followed - by the addition of 0.01N NaOH using a Radiometer pH Stat assembly (Radiometer, Copenhagen; pH meter type 26 utilising a glass and a calomel electrode, Titrator type 11, Titrigraph type SBR 2c and syringe burette type SRU 1a). When no

indication/

indication of proteolytic activity was apparent upon the addition of further small aliquots of enzyme the insoluble material was collected by centrifugation and washed with 5M guanidine hydrochloride in 0.1M Tris (pH 7.4) to remove any inhibitory digestion products. After further washing in 2M NaCl and water the residue was re-suspended in 0.01M $CaCl_2$ and the treatment with collagenase repeated. When there was no evidence of any digestion the purified elastin was collected and washed with 5M guanidine hydrochloride in Tris (pH 7.4), 2M NaCl and water before being lyophilised.

b) Autoclaving (15)

Bovine ligamentum nuchae, defatted in chloroform-methanol (3: 1 by volume) was finely powdered in a hammer mill, the 100 - 200 mesh fraction being collected. The powder was suspended in distilled water and autoclaved at a pressure of 15 pounds per square inch for sixty minutes. The residue was collected by filtration and washed with hot distilled water. The protein content of the filtrate was estimated by the Folin-Lowry method and the autoclaving repeated until no further protein was solubilised. The washed residue was then lyophilised.

c) Hot Alkali Treatment (17)

Defatted bovine ligamentum nuchae was powdered in a hammer mill and the 50 - 100 mesh (For use in the purification of collagenase by affinity chromatography) and 100 - 200 mesh fractions collected.

The two fractions were suspended in 0.1N NaOH which was boiled under reflux for 45 minutes. The suspension was cooled to room temperature, neutralised with 1N HCl, and the insoluble material collected by filtration, washed exhaustively with water and lyophilised.

a) The Isolation of Insoluble Elastin From Bovine Aorta Collagenase in conjunction with Guanidine Hydrochloride and Dithiothreitol

Aortae were removed from the carcasses of three-year old cattle within a few hours of the death of the animals. The tunica media was isolated by dissection from the descending aorta and divided into small pieces which were homogenised in ice-cold 0.1M Tris (pH 7.4) using a Waring blender. The homogenate was drained and suspended in 5M guanidine hydrochloride (62) in 0.1M Tris (pH 7.4) and the suspension stirred at 4 °C for 24 hrs. The insoluble material was then collected by centrifugation, and resuspended in fresh 5M guanidine hydrochloride in Tris. The extraction was continued for a total of ten 24 hr periods after which the residue was collected and suspended in 5M guanidine hydrochloride in 0.1M Tris (pH 7.4) containing 0.4% EDTA for treatment with dithiothreitol. Before addition of dithiothreitol, to a concentration of 0.05M,

the/

the suspension was degassed under reduced pressure and placed under a nitrogen barrier. The extraction was allowed to proceed for 24 hrs at 37 °C before the residue was collected by centrifugation, washed with 5M guanidine hydrochloride in Tris containing EDTA, and subjected to a further 24 hr extraction with dithiothreitol. The extracted material was then collected and washed with 5M guanidine hydrochloride in Tris followed by 2M NaCl and H_2O prior to lyophilisation.

Aliquots of the lyophilised material were then treated with collagenase which had been purified by affinity chromatography. As in the case of the material from ligamentum nuchae the digestion was performed in 10mM $CaCl_2$ at an enzyme to residue ratio of approximately 1: 1000, using a Radiometer pH Stat assembly to maintain the pH at a value of 7.5 during the digestion. When digestion was complete (after approximately 8 - hrs at 37°C) and addition of a small amount of collagenase produced no evidence of further digestion, the residue was collected by centrifugation and washed with 5M guanidine hydrochloride in 0.1M Tris (pH 7.4), 2M NaCl and water. The washed residue was then treated with collagenase under the same experimental conditions as before. When there was no indication of digestion, the insoluble material was collected, washed with guanidine, NaCl and water, and lyophilised.

b) Hot Alkali Treatment (17)

A sample of aortic elastin, purified with guanidine,

dithiothreitol/

dithiothreitol and collagenase as described above, was subjected to treatment with hot alkali in order to determine both the quantity of material soluble under these conditions and the effect of this widely used purification procedure upon the chemical integrity of the protein.

A known weight of elastin was suspended in 0.1N NaOH which was boiled under reflux for 45 minutes. The suspension was cooled to room temperature and neutralised with 1NHCl. The insoluble material was collected on a weighed 'Teflon' filter, thoroughly washed on the filter with distilled water and then dehydrated with ethanol, acetone and finally diethyl ether. The weight of the air-dried residue and filter was then determined.

Chemical Analyses

a). Amino Acid Analysis

Samples for amino acid analysis were hydrolysed in constant-boiling HCl ('Aristar' grade, B.D.H.) (2 ml acid per 1 mg sample) at 110 °C under nitrogen in sealed tubes for 24, 36 and 72 hrs. Only that part of the tube containing liquid was immersed in the oil-bath, thus allowing reflux from the upper walls. Samples which proved to have a tendency to char were dissolved by heating the tube in a hot water bath before hydrolysis at 110°C. Hydrolysates were

taken/

taken to dryness by rotary evaporation at 40°C and left overnight under reduced pressure in the presence of NaOH pellets. Amino acid analyses were performed on both a Jeol JLC - 6AH and a Locarte (single column operation) amino acid analyser. Standard colour values for the majority of amino acids were obtained from the chromatography of suitable volumes of a standard solution of amino acids (Sigma (London) Chemical Co., Ltd.) Amino Acids were quantitated manually from chromatograms, rather than by the use of an integrator.

Hydroxyproline was also estimated independently on protein hydrolysates by the colorimetric procedure of Blumenkrantz and Asboe-Hansen (63), in which hydroxyproline is oxidised by periodic acid in a strongly acid medium. The chromogen so formed is extracted from the reaction mixture with organic solvents and coupled with p - dimethylaminobenzaldehyde to produce a stable chromophore with an absorption maximum at 565 nm. Neither proline nor hydroxylysine are oxidised in the acid medium and consequently produce no interference.

b) Estimation of Hexosamines

For hexosamine estimation samples were hydrolysed in 4N HCl (2 ml per 1 mg of sample) under nitrogen in sealed tubes at 110°C. After 8 hrs the pH of the hydrolysate was adjusted to a value of 9 with NaOH solution and the total hexosamine content determined according to the procedure of Cessi and Piliego (64), in which the

reaction/

reaction of liberated hexosamine with acetyl acetone at pH 9.8 yields a volatile chromogen which is distilled from the reaction mixture to give a chromophore (absorption maximum at 545 nm) on reaction with p - dimethylaminobenzaldehyde. This method is insensitive to interference by neutral sugars as the chromogens formed by amino acids in the presence of glucose are non-volatile.

Solutions of galactosamine hydrochloride and glucosamine hydrochloride (Sigma (London) Chemical Co., Ltd.) were used to prepare standard curves.

Galactosamine and Glucosamine were also quantitated on a Locarte amino acid analysor (single column operation, when they were eluted in the region of tyrosine and phenylalanine). Hydrolysis of samples was performed in 4N HCl at 110°C for 8 hrs in sealed tubes under nitrogen. Hydrolysates were taken to dryness by rotary evaporation at 40°C and stored overnight at reduced pressure over NaOH pellets before analysis. Standard chromatograms were obtained using suitable volumes of a solution of galactosamine hydrochloride and glucosamine hydrochloride of known concentration.

c) Estimation of Neutral sugars

Neutral sugars were determined by both a gas chromatographic and a colorimetric technique.

i) Gas Chromatography

It was found that if a whole protein-hydrolysate was subjected to the various preparative procedures necessary to form

volatile/

volatile derivatives of hexoses suitable for gas chromatographic estimation serious interference was produced by the amino acids. To overcome this, the procedure of Anastassiadis and Common (65) was adopted to remove both amino acids and hexosamines from the protein hydrolysate.

Samples for hexose determinations were placed in thick-walled glass tubes to which was added 10 ml of a 20% (weight by volume) suspension of Dowex AG 50W x 8 (H + Form) (Bio-Rad Laboratories) in 0.05 NHCl. Hydrolysis was performed at 105°C for 24 hrs after which the suspension was quantitatively transferred to a glass column (1.2 cm x 30 cm) with a glass sinter at the lower end. Neutral sugars were eluted with 50 ml H_2O; no attempt was made to isolate the hexosamines retained on the column. The effluent was taken ty dryness by rotary evaporation at 40°C.

The isolation of neutral sugars from similar samples was also performed by an alternative procedure (66), the protein being hydrolysed in 6 ml of 0.5N H_2SO_4 for 15 hrs at 100°C. H_2SO_4 was then neutralised by the addition of a saturated solution of Ba (OH_2) and the resulting precipitate of $BaSO_4$ removed by low speed centrifugation. The clear supernatant was applied under gravity to a column (1.0 cm x 7.0 cm) of Dowex AG 50W x 1 (H + Form) (Bio-Rad Laboratories) equilibrated and eluted with H_2O. Neutral sugars were eluted in the first 20 ml, which were taken to dryness

by/

by rotary evaporation.

Sugars were estimated according to the procedure of Bhatti et al. (67). Hexoses from the sample were first converted, together with a known amount of perseitol as an internal standard, to their methyl glycosides, from which volatile derivatives suitable for gas chromatographic analysis were formed by trimethylsilylation.

The gas chromatograph used was a Pye series 104 instrument equipped with hydrogen flame ionisation detectors and coupled to a Philips 8000 recorder. Dual column operation was used, the glass chromatographic columns (270 cm x 0.6 cm) being packed with 3.8% of SE ± 30 on Diatoport S (Hewlett-Packard Ltd., Slough, Bucks).

Operating conditions were:-

Carrier gas: Argon (45 ml/ minute)

Column temperature: programmed from 140 °C to 200°C at 0.5°C/minute, the upper limit being held until the last peak emerged.

A standard solution of various sugars were prepared for derivatisation and gas chromatography and allowed the identification and quantitation of hexoses from protein samples.

ii). Colorimetric Analysis

The anthrone technique (68) was used for the colorimetric determination of hexoses. As amino acids can cause interference in

this/

this procedure, neutral sugars were again isolated by the method of Anastassiadis and Clamp (65). Standard curves were prepared using solutions of either glucose or galactose of known concentration.

d) Amino end-group Analysis

i) 2 - chloro - 3,5 - dinitropyridine method.

A modified version of the procedure described by Signor et al (69) was adopted for the estimation of N - Terminal residues in elastin. Preliminary experiments indicated that diffusion of reagent into the interior of protein particles became the rate limiting step of the condensation if material with a particle size greater than 150 microns was subjected to amino end-group analysis. As very finely powdered elastin proved inconvenient to handle the 100 - 200 mesh fraction (79 - 149 micron particle diameter) was selected for use in N-terminal determinations. As 2 - chloro - 3,5 - dinitropyridine spontaneously hydrolyses under the experimental conditions used in the condensation procedure the reagent was added in two aliquots to the protein, with a period of 8 hrs between the additions.

An aliquot (100 mg) of protein was suspended in 6 ml of 50% (volume by volume) ethanol containing 2.5% $NaHCO_3$. When the protein was fully equilibrated 1.5 ml of the reagent solution - 2-Cl-3,5 - dinitropyridine (B.D.H), 2% weight by volume in ethanol - was added and the suspension shaken in the dark at room temperature. After 8 hrs

a/

a further 1.5 ml aliquot of the reagent solution was added and the reaction allowed to proceed for an additional 16 hrs. The reaction mixture was then acidified (pH 2-3) with 1N HCl, the ethanol removed by rotary evaporation, and dinitropyridyl - protein recovered by centrifugation, washed with ethanol and air dried. The sample was then hydrolysed in 3N HCl containing 49% Formic acid (by volume) for 45 minutes at 100°C, when the dinitropyridyl protein completely dissolved. The hydrolysate was taken to dryness by rotary evaporation at 40°C and left in the presence of pellets of NaOH for 16 hrs in the dark under reduced pressure. Water (6 ml) was added, the pH adjusted to a value of 2 and the solution extracted six times with one volume of ethyl acetate. The pooled extracts were dehydrated over anhydrous Na_2SO_4, filtered and evaporated to dryness. The residue was dissolved in 1 ml of 30% (weight by volume) NH_4OH and, with the aid of four 1 ml washes of NH_4OH, quantitatively transferred to a thick walled glass tube. Loss of ammonia from the NH_4OH solution was minimised during these operations by chilling the glassware in ice-baths. The solution of dinitropyridyl amino acids in NH_4OH was frozen in liquid nitrogen and the tube carefully sealed under reduced pressure. Regeneration of free amino acids from their dinitropyridyl derivatives was performed in the dark at 100°C for 35 minutes (70). The tube was then cooled, opened and the solution quantitatively transferred to a round-bottomed flask and taken to dryness by rotary evaporation.

The/

The residue was dissolved in 20 ml H_2O and reaction by-products removed by extraction with ethyl acetate, firstly at neutral pH and then at pH 4. The extractions were continued until a colourless extract was obtained, and the extracted solution then taken to dryness.

Free amino acids were identified and quantitated on a Locarte amino acid analyser (single column operation).

To establish whether all free N-terminal groups located within the compact structure of the protein had reacted with 2 - Cl - 3,5 - dinitropyridine, an aliquot (100 mg) of elastin (isolated with collagenase purified by chromatography on G200 and DEAE cellulose) was equilibrated with dimethyl sulphoxide - 5% $NaHCO_3$ (1:1 by volume). 2 - Cl - 3,5 - dinitropyridine was then added to give a 0.1% solution (weight by volume) and the reactions allowed to proceed for 2 hrs in the dark at room temperature. The recovery of dinitropyridyl - elastin, the isolation of dinitropyridyl amino acids, and their regeneration and quantitation were carried out as previously described.

ii). Cyanate Procedure

As a check on the efficiency of the 2 - chloro - 3,5 - dinitropyridine method of amino end-group estimation a sample of elastin, prepared by the use of collagenase purified by chromatography on G200 and DEAE cellulose, was subjected to N-terminal

analysis/

analysis by a modified version of the cyanate procedure of Stark and Smyth (71). In a preliminary experiment an attempt was made to determine the N-terminal profile of elastin using the cyanate procedure exactly as described by Stark and Smyth. In this method the free amino functions of a protein are reacted with cyanate to yield a carbamyl protein. Upon heating the carbamyl protein in strong acid, the carbamyl groups cyclise to the corresponding hydantoins, with accompanying cleavage of the peptide bond. The resulting mixture of hydantoins, amino acids and peptides can be fractionated by passage through a column of the hydrogen form of a strongly acidic ion-exchanger such as Dowex 50, when amino acids, peptides and the hydantoins of basic amino acids (histidine and arginine) are retained while the hydantoins of other amino acids appear at the break through point. Amino acids are regenerated in nearly quantitative yields on heating the hydantoins with 0.2 N NaOH. However, in the case of elastin it was found that the hydantoins eluted from the column of Dowex 50 were contaminated by an acidic peptide(s) particularly rich in glutamic acid. In the procedure finally adopted this contaminant was removed by an additional fractionation stage on Sephadex G-15.

Two ml each of N- ethylmorpholine (colourless, redistilled preparation) and water were mixed and the pH of the mixture adjusted to 8 with glacial acetic acid. To this buffer were added 2.9 g of solid, recrystallised guanidine hydrochloride (62) and the final volume adjusted to 5 ml with water. An aliquot (100 mg) of elastin

was/

was suspended in 5 ml of 6M guanidine hydrochloride in a 'Teflon' capped hydrolysis tube, to which was added the 5 ml of buffer. followed by 500 mg of KCNO. The carbamylation reaction was allowed to proceed to 50°C overnight with continuous stirring. Glacial acetic acid (10 ml) was slowly added with stirring to the reaction mixture in order to decompose excess cyanate into carbon dioxide and ammonia. The carbamylated elastin was collected by centrifugation and thoroughly washed, first with distilled water, then acetone and finally air-dried. To the dried carbamylelastin was added 3 ml each of 50% acetic acid and concentrated hydrochloric acid, the mixture gassed out with nitrogen and the tube tightly capped. After cyclisation at 100°C for 1 hr the contents of the tube were evaporated to dryness and 0.01 N HCl (1 ml) added to the residue.

A column of Dowex 50 W x 2 (H^+ Form, 100 - 200 mesh, Bio-Rad Laboratories) was prepared in a glass tube (1.0 x 48 cm) fitted with a coarse glass-sinter. The solution of hydantoins, amino acids and peptides was placed on the column with the aid of three 1 ml rinses of 0.01 N HCl. The column was then developed with 0.01N HCl at 18°C and a flow rate of 36 ml/h. The effluent was monitored by scanning ultraviolet spectrophotometry and the first 72 ml collected and evaporated to dryness. The residue, consisting of hydantoins together with a contaminating peptide(s) , was re-dissolved in 3 ml of 0.02N NH_4OH and loaded on to a column (1.6 x 95 cm) of Sephadex G-15 equilibrated and eluted with 0.02N NH_4OH

at/

at a flow rate of 7 ml/h at 18°C. As before, the column effluent was monitored by scanning ultraviolet spectrophotometry and the hydantoins collected between 130 ml and 150 ml (Void volume = 82 ml). The pooled fractions were taken to dryness and the free amino acids regenerated in 2 ml of 0.2 N NaOH at 110 C for 20 hrs. After the regeneration NaOH was neutralised with 1N HCl and the solution evaporated to dryness. Amino acids were then identified and quantitated on a Locarte amino acid analyser (single column operation).

e). Estimation of Acetyl Groups

A gas chromatographic procedure was adopted for the detection and estimation of acetyl groups in elastin isolated from bovine ligamentum nuchae by the use of collagenase, purified by affinity chromatography, in conjunction with guanidine and dithiothreitol.

Although 6N HCl may be used to convert protein acetyl groups to acetic acid (72) H_2SO_4 has the advantage that many proteins, difficult to dissolve in HCl, often prove easily soluble in 12N H_2SO_4, which may then be diluted to 6N. As rather large aliquots (40 mg) of elastin (100 - 200 mesh fraction) were to be hydrolysed this technique was adopted and the protein was first dissolved in warm 12N H_2SO_4 (0.25 ml) to which was then added 0.25 ml H_2O. In this way it was possible to hydrolyse the protein in small volumes of acid with no occurrence of charring. Hydrolysis was performed under nitrogen in sealed tubes at 108°C for 16 hrs. (73). The tube was positioned such that only the liquid containing portion was submerged in the oil bath, thus allowing reflux from the upper walls.

After/

After hydrolysis, the tube was cooled in an ice-bath and centrifuged to remove liquid droplets from the walls. 100 mg of anhydrous Na_2SO_4 and 0.5 ml of tert-butylethyl ether (Eastman Kodak) were then added. The extraction of acetic acid into the ether was facilitated by stirring of the mixture on a vortex-stirrer. The tube was then centrifuged at low speed in order to obtain a clear separation of the aqueous and organic phases. The tert-butylethyl ether was removed with a Pasteur pipette which had been drawn to a fine tip and the ether transferred to a small, stoppered vial containing 300 mg of anhydrous Na_2SO_4. Two more extractions were performed and the pooled extracts dried over Na_2SO_4 for approximately 30 minutes prior to gas chromatography (72).

The gas chromatography was performed with a Pye series 104 instrument equipped with a hydrogen flame ionisation detector and coupled to a Philips PM 8000 recorder. The glass chromatographic column (0.6 cm x 150 cm) was packed with Porapak Q (Waters Assoc., Inc., Framingham, U.S.A.), a polyaromatic polymer in beaded form, 100 - 120 mesh, prepared from ethylvinylbenzene with divinylbenzene as a cross-linker. The beads were washed on a sintered-glass funnel with ethanol and coated with a chloroform solution containing 10 g of carbowax 20M - terephthalic acid per 100 ml (73). Operating conditions were:

Carrier gas: Argon (45 ml/ minute)

Column temperature: 192°C (the column was conditioned at 230°C for 1 hr.)

Detector/

Detector cell temperature: 240°C

Reference standards were prepared by diluting measured volumes of glacial acetic acid into tert-butylethyl ether so that 4 -5 μl of injected sample contained the desired amount of acetic acid. Ovalbumin (Sigma (London) Chemical Co., Ltd.) was used as a test protein.

This procedure was also suitable for the estimation of propionyl and butyryl groups in the protein. Formyl groups could not, however, be estimated as formic acid coincided with a component of the tert-butylethyl ether solvent.

Removal of N-Terminal Blocking Groups

a). Treatment of Elastin with Sodium Hydroxide, Under Mild Experimental Conditions.

In an attempt to free the N-terminal residue of mature, insoluble elastin from possible alkali-labile groups with the minimum rise in the background level of amino-end groups, samples of both aortic and nuchal ligament elastin (isolated by the use of collagenase, purified by affinity chromatography, in conjunction with guanidine and dithiothreitol) were subjected to the action of dilute alkali under mild experimental conditions.

Aliquots of elastin were suspended in dioxan-water (3:1 by volume) containing 0.25N NaOH and stirred for 1 hr at 18°C. The

residue/

residue was collected by filtration, suspended in water and neutralised with 1N HCl, washed with water and lyophilised.

Similar samples of ligament elastin were treated in the same way with a more concentrated solution of NaOH in aqueous dioxan (0.5N NaOH in dioxan - H_2O, 1:1 by volume).

b) Treatment of Elastin with Methanolic Hydrogen Chloride

In an attempt to remove possible acid-labile masking groups from the N-terminal residue of mature, insoluble elastin the protein was exposed to the action of HCl under mild experimental conditions, chosen so as to minimise the cleavage of peptide bonds by the acid and the accompanying increase in the background level of amino-end groups.

Samples of ligamentum nuchae elastin (isolated by the use of collagenase, purified by affinity chromatography, in conjunction with guanidine and dithiothreitol) were suspended in methanol ('Aristar' grade, B.D.H. The methanol was dried by refluxing over calcium oxide for several hours) containing anhydrous hydrogen chloride at a concentration of 1.5N. Suspensions were magnetically stirred in vessels equipped with water traps for periods ranging from 30 to 240 minutes. After treatment with methanolic - HCl residues were collected on 'Telflon' filters and thoroughly washed with dried methanol until the pH of the washings, when diluted with an equal volume of water, had a value no lower than 5. The elastin samples were then air-dried.

Similar/

Similar aliquots of elastin were suspended in methanol, which had not been dried over calcium oxide, ('Aristar' grade methanol, B.D.H. Water content approximately 1000 ppm), containing 1.5N HCl and stirred for 60 minutes at 26°C and then collected, washed and dried as before.

Amino-end group determinations were performed on both alkali and acid treated elastin samples, using the 2 - chloro - 3,5 - dinitropyridine method.

Electron Microscopy

Elastin which had been isolated from bovine ligamentum nuchae by the use of collagenase, purified by affinity chromatography, in conjunction with guanidine and dithiothreitol was finely powdered in a vibrating agate ball-mill (Vibromill, RIIC) for thirty 1-minute periods. Aliquots were then subjected to one of the following treatments:

a). Elastin (2 mg) was suspended in 2 mls of 12 mM uranyl formate - 10 mM oxalic acid (adjusted to pH 6.4 with ammonium hydroxide) (74) and spread onto carbon-coated grids.

b). To facilitate diffusion of the stain into the interior of the specimen, elastin was swollen for 10 minutes in dimethyl sulphoxide (59,60) containing 1% uranyl formate. Ten volumes of 12 mM

uranyl/

uranyl formate - 10 mM oxalic acid (pH 6.4) were added and after 1 hr., elastin collected by centrifugation, washed with and re-suspended in buffered uranyl formate and finally spread onto carbon-coated grids.

c). A suspension of elastin in water was subjected to ninety 1 - minute periods of sonication (Mullard - MSE 60 watts, ~ 20 kc), the temperature being controlled by imersion in an ice-bath. The protein was collected by centrifugation, suspended in buffered uranyl formate solution and spread onto carbon-coated grids.

Grids were examined in an AEI EM 6B electron microscope. Micrographs were taken at instrumental magnifications of 40,000, 60,000 and 100,000, calibrated using a diffraction-grating replica.

Investigation of the Mechanical Properties of Ligamentum nuchae and purified elastin samples.

a). Preparation of Samples

Samll strips of tissue were gently dissociated from untreated bovine ligamentum nuchae to give specimens of fairly uniform diameter, between 1 mm and 2 mm, and of lengths greater than 1 cm. A selection of the strips was then washed in 1% NaCl for 24 hrs at 4°C in the presence of a few drops of toluene, followed by six 24 hr

extractions/

extractions at 4°C in 5M guanidine hydrochloride in 0.1M Tris (pH 7.4) and two 24 hr extractions at 37°C in 5M guanidine hydrochloride in 0.1M Tris (pH 7.4) containing 0.05M dithiothreitol and 0.4% EDTA. After thorough washing with water the strips were treated with collagenase (purified by affinity chromatography). The digestion was performed in 0.1M Tris (pH 7.5) containing 0.01M $CaCl_2$ at an enzyme to sample ratio of approximately 1:1 000 and at a temperature of 37°C for two 24 hr periods. The flasks were shaken rather than stirred in order to prevent any mechanical damage to the strips and toluene was added to inhibit bacterial action. After treatment with collagenase the strips were washed with 5M guanidine in Tris followed by water and air-dried.

A number of strips were withdrawn after extraction with dithiothreitol and were not treated with collagenase; also several strips were treated with collagenase but were not extracted with guanidine or dithiothreitol.

Each end of the air-dried strips was embedded in epoxy resin and attached at the same time to a short length of fine, single-strand copper wire. After equilibration in water for 18 hrs the dimensions of each strip were determined with a travelling microscope. The diameter, at 2 mm intervals, and the length of the strip were measured and the strip then rotated on its long axis through 90° and the measurements repeated. In the calculation of stress and Young's Modulus for each strip the average of these diameter measurements was used.

b)./

b). Dynamometry

Strips were carefully selected for straightness and uniformity of diameter. One end of each strip was connected, by means of a metal chain, to the shaft of a multi-turn precision helipot (Beckman Instruments Ltd.,) while the other end was attached to a microdisplacement myograph transducer (Type F 10 000, E and H Instrument Co., Inc., Houston, U.S.A.). The outputs of the helipot and transducer were fed into the X and Y channels, respectively, of an XY recorder (Bryans XY/T autoplotter series 22000, model 22020). This arrangement resulted in a direct plot of tension and variation of specimen length when the helipot shaft was rotated. Experiments were performed in water at 20°C, under thermostatic control.

X-Ray Analysis

Purified elastin strips were prepared and mounted on copper wire, as described for mechanical tests, and equilibrated in water overnight. Orientated specimens were obtained by extending strips by either 40% or 65% of their original relaxed length using a sliding metal frame. Strips were air-dried on the frame and, on removal, retained the extension indefinitely, providing they were stored in the presence of a dessicant, such as silica gel.

X-Ray/

X-ray studies were carried out on both extended and unextended samples. Wide angle and small angle X-ray scattering patterns were obtained using a Warhus camera with 0.015 inch diameter pin-holes and film to sample distances of 48 mm and 308 mm respectively. The camera was evacuated during exposures and the scattering patterns obtained with nickel-filtered Cukα radiation. An exposure time of 12 hrs was sufficient for wide angle scattering patterns but 3 days were necessary for small angle exposures.

X-ray diffraction patterns were obtained by Dr. B. Delf of the Department of Physics, University of Cardiff.

Optical Polarisation Analysis

a). Both longitudinal and transverse sections, 25 microns in thickness, were cut from elastin strips (purified as described for mechanical tests) on a cryostat. The optical character of the protein was investigated by examination of these sections under a Leitz polarising microscope equipped with a Berek compensator (Leitz).

b). A small portion of a purified elastin strip was equilibrated in water and gently teased apart under a dissecting microscope, until the small fibres (approximately 10 microns in diameter) of

which/

which the material is composed were separated. The fibres were dried and then immersed in liquids of known refractive index. The dependence of the relative retardation of the fibres upon the refractive index of the surrounding medium was determined using a Leitz polarising microscope equipped with a Berek compensator.

RESULTS

Purification of Collagenase

a). Gel and Ion-exchange Chromatography.

The elution profile of the crude collagenase preparation (400 mg dissolved in 30 ml 5mM Tris (pH 7.5) -4mM $CaCl_2$) on Sephadex G200 (5 x 100 cm column) is reported in Fig.2. The brown pigment, present as a contaminant in the unpurified enzyme preparation, migrated down the chromatographic bed as a single broad band which was not completely eluted until near the bed volume of the column. A clearly separated peak at the void volume was evident when the fractions were monitored by ultraviolet spectrophotometry, but proved to possess neither caseinolytic nor collagenolytic activity. Collagenolytic activity was present in only one peak, that occurring as a shoulder on the large peak representing the brown pigment. Material with caseinolytic activity was eluted only slightly later than the collagenolytic activity and some overlap occurred between the two. No peak coincident with the caseinolytic activity was evident upon examination of the fractions by ultraviolet spectrophotometry.

Those fractions indicated in Fig.2 as lying between the two arrows were pooled and stored at 4°C until the corresponding fractions from two more fractionations on G200 had been collected. The three sets of pooled fractions were combined and applied to a column of DEAE cellulose. As previously described the column was eluted with various buffer solutions in a step-wise manner,

and/

Fig. 2. The elution profile of crude collagenase on Sephadex G-200.

Load: 400 mgs in 30 ml

Buffer: 5 mM Tris (pH 7.5) - 4 mM $CaCl_2$

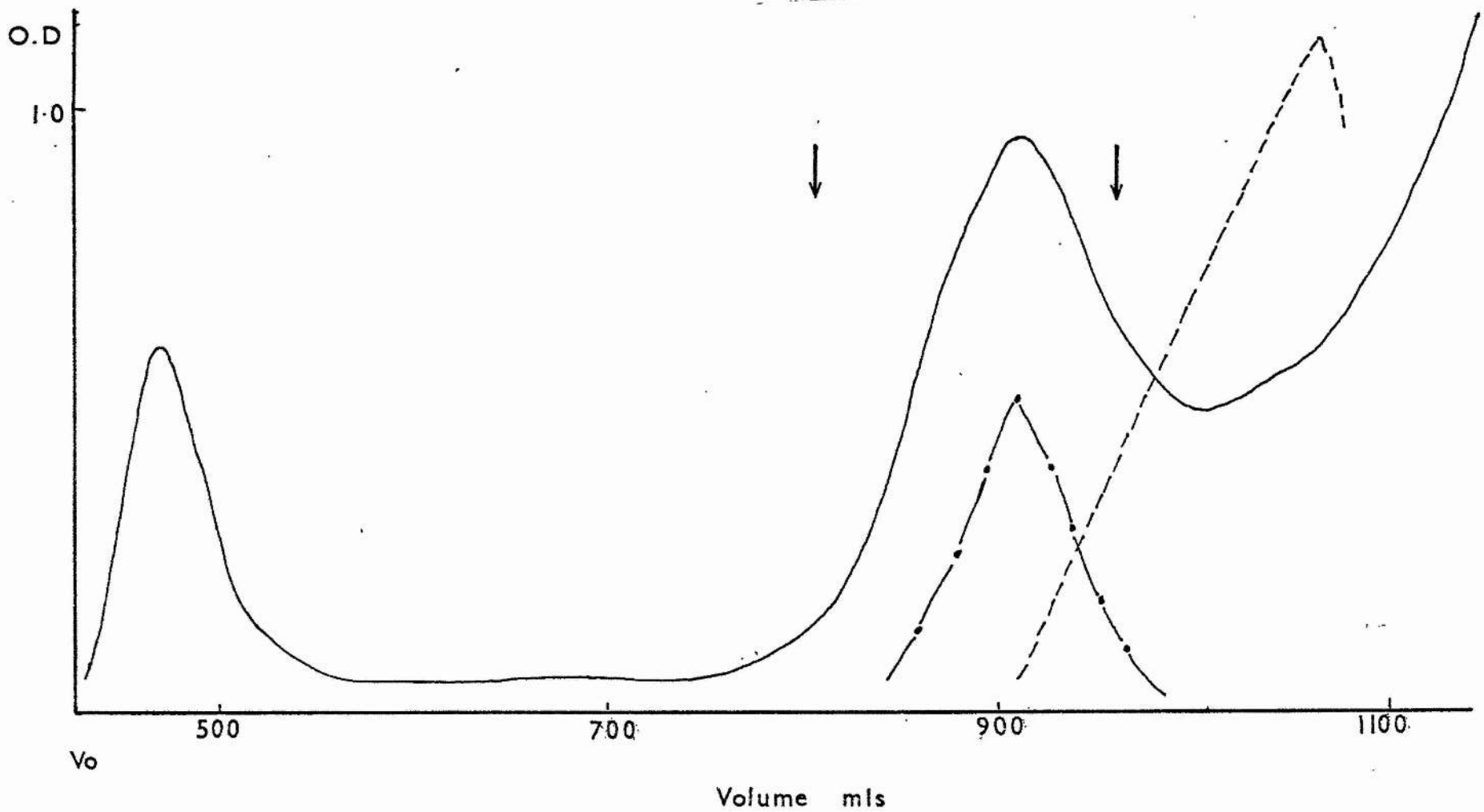
Flow Rate: 22 ml/h

Column size: 5 x 100 cms

_____ O.D. 278 nm

. _____ . O.D. 578 nm : Collagenolytic Activity.

--------- O.D. 578 nm : Caseinolytic Activity


O.D
1·0
500
700
900
1100
Vo
Volume mls

and the elution profile and buffer changes are shown in Fig.3. No buffer changes were made until a stable baseline reading of optical density at 278 nm was obtained. The contaminating brown pigment was seen to form a narrow band at the top of the column and was not removed by any of the buffer solutions.

The fractions of which a peak was composed were pooled, dialysed against 0.1 mM $CaCl_2$ and lyophilised. The lyophilised material was then assayed for activity towards collagen and elastin, and the results of these assays are summarised in Table 3.

Table 3 : Activity of DEAE cellulose fractionated material. (expressed as μmoles Leucine equivalents released per hour per mg protein at 37°C in the presence of excess substrate).

Substrate:	Calf skin collagen	NaOH purified elastin
Peak 1	4.3	0.50
Peak 2	6.3	0.05
Peak 3	4.3	0.03
Peak 4	0.6	0.01

The material from peaks 2 and 3 was selected for use in the purification of elastin.

b). Affinity Chromatography

The initial purification of the crude collagenase solution

with/

Fig. 3. The elution profile of partly purified collagenase on DEAE - Cellulose.

Flow Rate : 40 ml/h

Column size : 2 x 10 cm

Buffers : a 5 mM Tris - 4 mM $CaCl_2$

b 50 mM Tris - 4 mM $CaCl_2$

c 5 mM Tris - 4 mM $CaCl_2$ - 100 mM NaCl

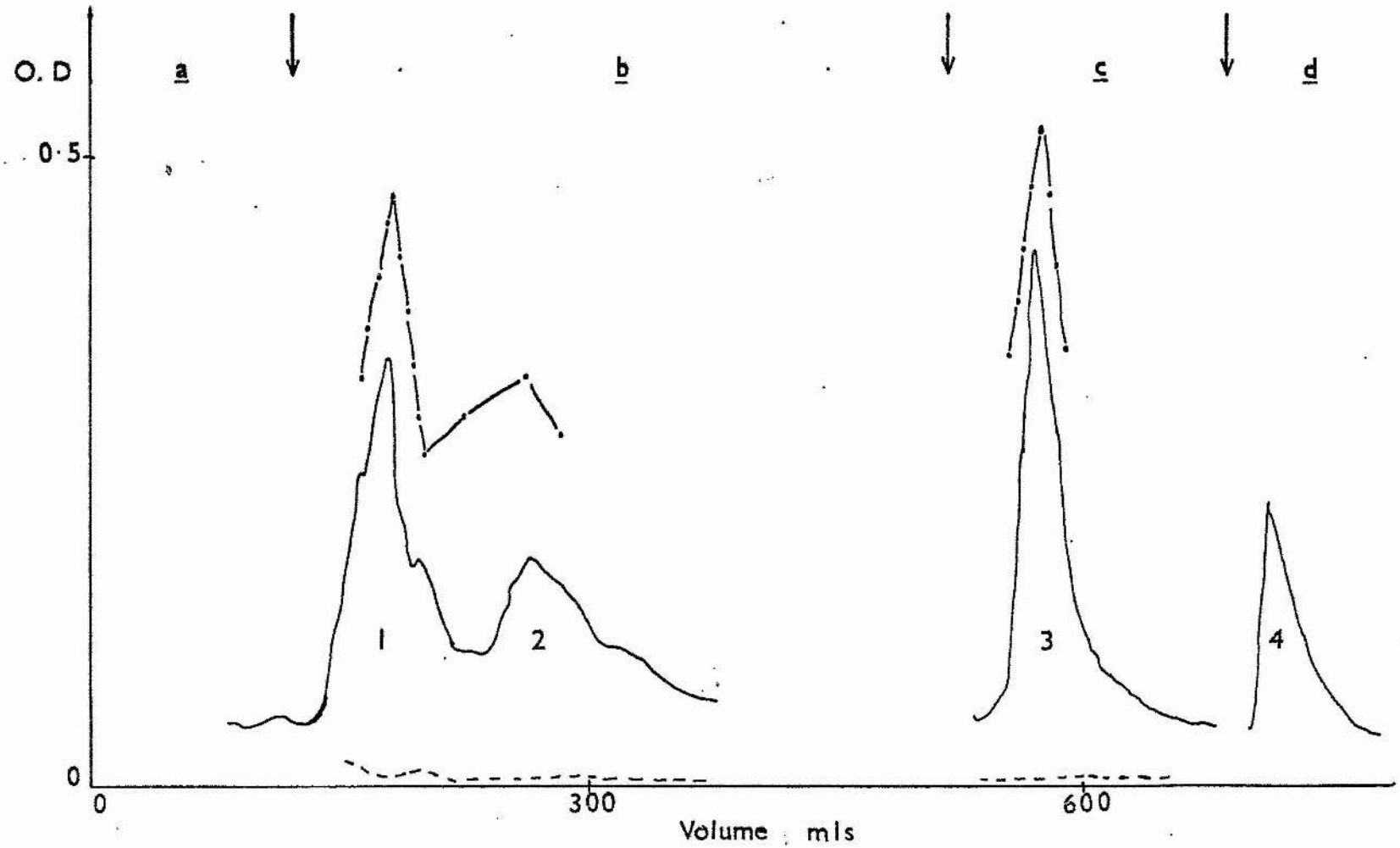
d 5 mM Tris - 4 mM $CaCl_2$ - 500 mM NaCl

All buffers pH 7.5

_____ O.D. 278 nm

. ____ . O.D. 578 nm : Collagenolytic Activity

-------- O.D. 578 nm : Caseinolytic Activity


O. D
0·5
0
a
b
c
d
1
2
3
4
0
300
600
Volume : mls

with DEAE cellulose yielded a slightly pigmented solution possessing both collagenolytic and elastolytic activity. As described earlier this solution was dialysed and lyophilised. The passage of aliquots of this material through a column of collagen-free elastin resulted in a preparation which was highly active towards collagen but in which no elastolytic activity could be detected upon prolonged incubation with insoluble elastin.

The Isolation of Elastin

The extraction of hexosamine and hydroxyproline containing material from bovine ligamentum was followed by estimating the amount of hexosamine and hydroxyproline in hydrolysates of dialysed extraction-supernatants. The results of these determinations are represented in Figs. 4 and 5. It can be seen that the majority of extractable material is solubilised and removed during the first 24 hours of extraction with 5M guanidine hydrochloride.

Fig. 4 The removal of hexosamine from bovine ligamentum nuchae by 5M Guanidine and Dithiothreitol in 5M Guanidine.

0 - 144 h : 5M Guanidinium hydrochloride - 0.1M Tris (pH 7.4)

144 - 192 h: 0.05M Dithiothreitol in 5M Guanidinium hydrochloride - 0.1M Tris (pH 7.4) containing 0.4% EDTA

Quantity of hexosamine removed is expressed as a percentage of the hexosamine present in defatted ligamentum nuchae.

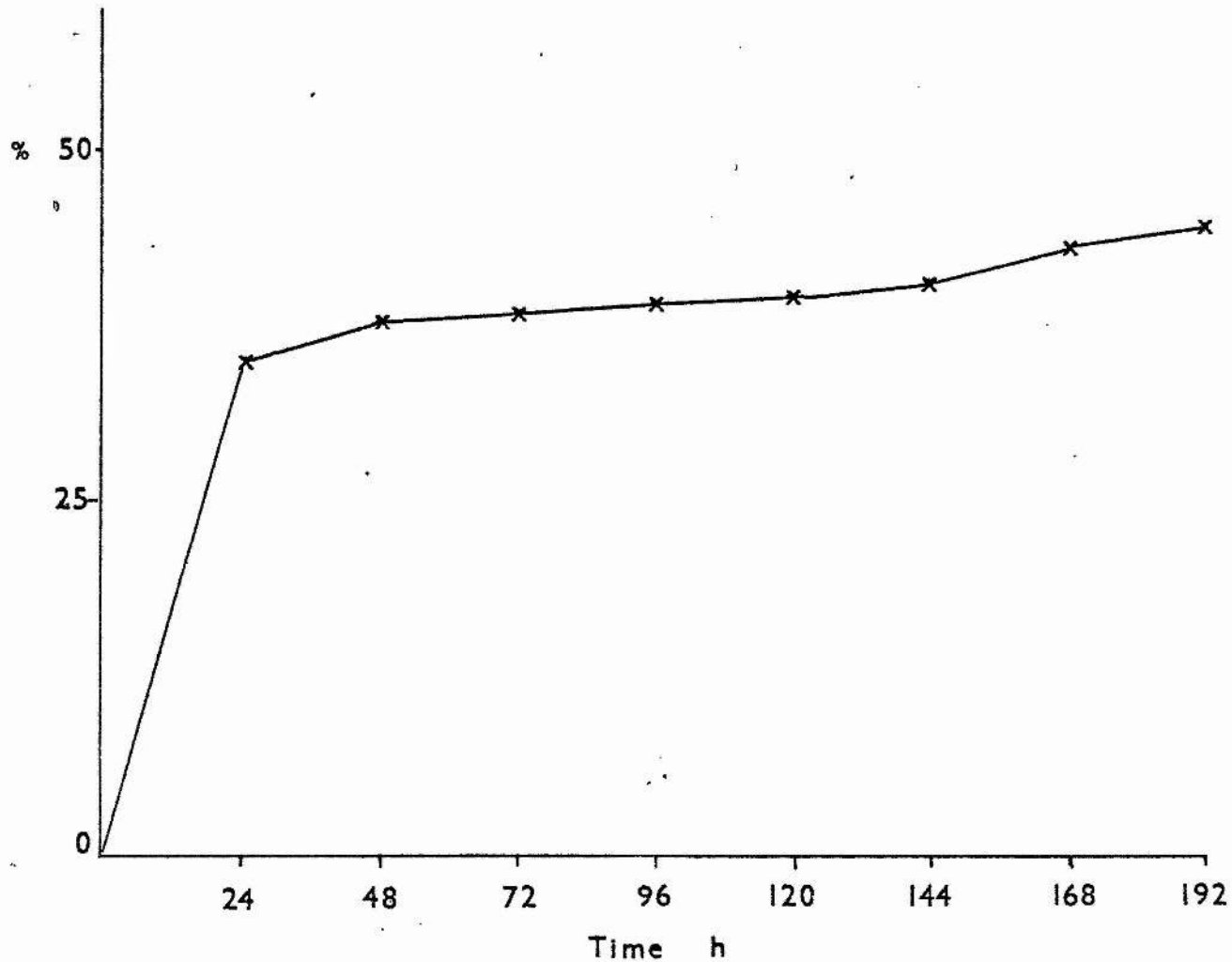
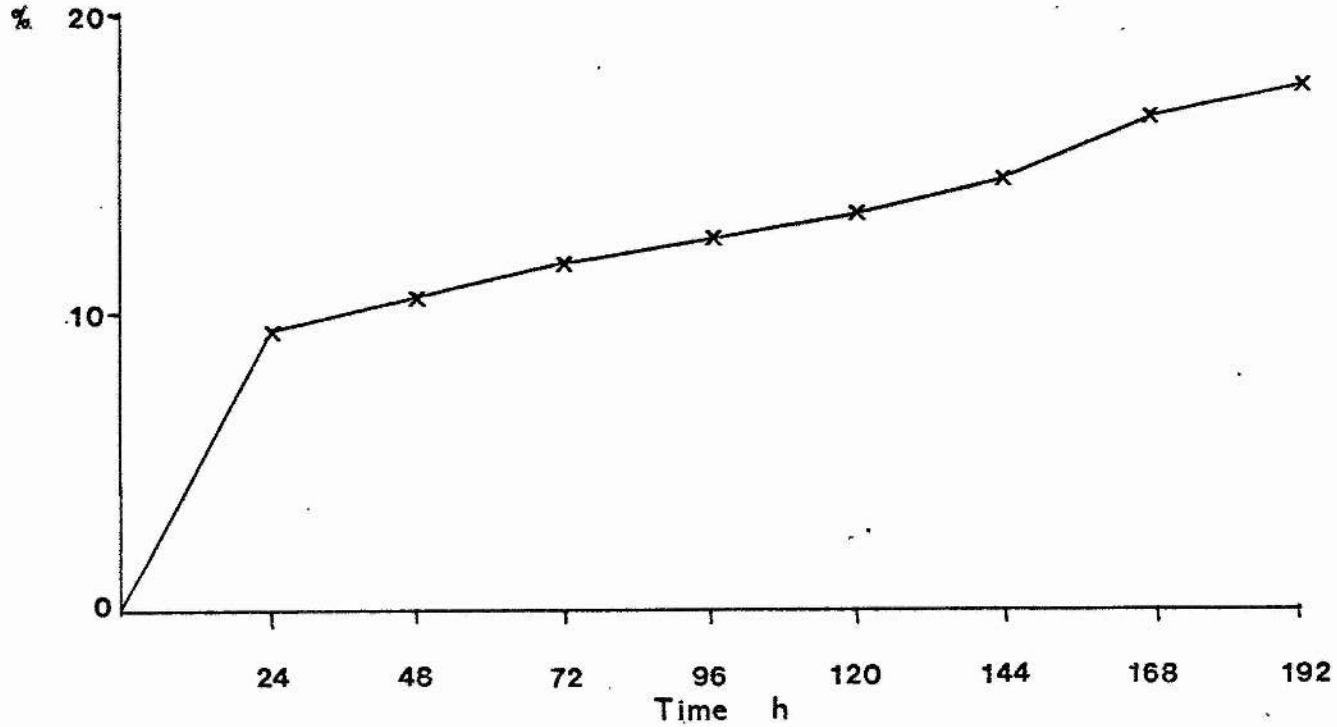

% 50
25
0
24
48
72
96
120
144
168
192
Time h

Fig. 5. The removal of hydroxyproline from bovine ligamentum nuchae by 5M Guanidine and Dithiothreitol in 5M Guanidine.

0 - 144 h : 5M Guanidinium hydrochloride - 0.1M Tris (pH 7.4)

144 - 192 h: 0.05M Dithiothreitol in 5M Guanidinium hydrochloride - 0.1M Tris (pH 7.4) containg 0.4% EDTA.

Quantity of hydroxyproline removed is expressed as a precentage of the hydroxyproline present in defatted ligamentum nuchae.


%
20
10
0
24
48
72
96
120
144
168
192
Time h

Chemical Analyses

a). Amino Acid Analysis

Tables 4,5,6 and 7 report the amino acid composition of defatted bovine ligamentum nuchae, the residues remaining after extraction with guanidine and dithiothreitol in guanidine and of enzymically purified elastin. Table 8 below traces the variation in concentration in the insoluble material of six amino acids during the isolation of elastin using collagenase in conjunction with guanidine and dithiothreitol.

Table 8 : Variation in the concentration of some amino acids during the isolation of Elastin from bovine ligamentum nuchae. (concentrations are expressed as residues per 1000 amino acid residues and have been corrected for hydrolytic losses).

	Defatted ligament	After Guanidine	After Dithrothreitol	After Collagenase
Hydroxyproline	26.3	20.2	21.3	8.1
Proline	112.7	111.0	111.0	116.1
Hydroxylysine	2.8	2.6	2.6	0.0
Isodesmosine *	4.4.	6.2.	5.3	6.2
Desmosine *	7.6	8.2	8.3	8.9
Half-cystine	18.3	2.2	1.9	0.0

* expressed as lysine residues.

Extraction/

Extraction with guanidine resulted in a dramatic decrease in the level of ½-cystine in the residue; subsequent treatment of the residue with dithiothreitol brought about only a small drop in ½-cystine concentration as the microfibrillar component was solubilised and removed. Sulphur-containing amino acids could not be detected in the residue after treatment with collagenase; hydroxylysine was also absent from the enzymically purified elastin.

For purposes of comparison, the amino acid composition of soluble elastin isolated from the ligamentum nuchae of copper deficient calfs (75) is reported in Table 9, while Tables 10 and 11 report the composition of elastin isolated from bovine ligamentum nuchae by autoclaving or hot alkali treatment. Both insoluble elastin, purified by collagenase, and its soluble precursor exhibit concentrations of acidic and hydroxy amino acids substantially higher than those shown by the NaOH treated preparation.

The amino acid composition of enzymically-purified insoluble aortic elastin is reported in Table 12, along with that of the same preparation after treatment with hot alkali. Exposure of aortic elastin to hot alkali resulted in a marked lowering of the concentration of four amino acids, namely aspartic acid, glutamic acid, threonine and serine.

The amino acid profiles of enzymically purified elastins from bovine ligamentum nuchae and aorta are very similar, the only significant differences being the concentrations of three residues, viz. hydroxyproline, lysine and tyrosine in the two preparations.

Table 4. The amino acid composition of defatted bovine ligamentum nuchae. (Values are expressed as residues/1000 amino acid residues and have been corrected for hydrolytic losses)

Hydroxyproline	26.3
Aspartic Acid	15.1
Threonine	12.6
Serine	13.4
Glutamic Acid	28.0
Proline	112.7
Glycine	328.2
Alanine	193.4
Valine	99.1
Half-cystine	18.3
Methionine	2.0
Isoleucine	23.0
Leucine	54.8
Tyrosine	6.0
Phenylalanine	26.6
Hydroxylysine	2.8
Lysine	7.7
Histidine	1.6
Arginine	13.1
Isodesomsine*	4.4
Desmosine *	7.6
Merodesmosine*	0.5
Lysinonorleucine *	2.6

*Expressed as lysine equivalents

Table 5. The amino acid composition of bovine ligamentum nuchae after extraction with guanidinium hydrochloride. (Values are expressed as residues/1000 amino acid residues and have been corrected for hydrolytic losses).

Hydroxyproline	20.2
Aspartic Acid	10.6
Threonine	9.7
Serine	11.7
Glutamic Acid	22.2
Proline	111,0
Glycine	338.7
Alanine	214.1
Valine	115.6
Half-cystine	2.2
Methionine	-
Isoleucine	4.1
Leucine	56.6
Tyrosine	5.7
Phenylalanine	27.8
Hydroxylysine	-
Lysine	6.0
Histidine	1.5
Arginine	9.9
Isodesmosine*	6.2
Desmosine *	8.2
Merodesmosine*	0.5
Lysinonorleucine*	2.7

* Expressed as lysine equivalents

Table 6. Amino acid composition of bovine ligamentum nuchae after extraction with guanidinium hydrochloride and treatment with dithiotheitol. (Values are expressed as residues/ 1000 amino acid residues and have been corrected for hydrolytic losses).

Hydroxyproline	21.3
Aspartic Acid	10.5
Threonine	9.6
Serine	11.3
Glutamic Acid	22.0
Proline	111.0
Glycine	340.5
Alanine	207.7
Valine	114.2
Half-cystine	1.9
Methionine	-
Isoleucine	23.5
Leucine	55.4
Tyrosine	6.6
Phenylalanine	27.5
Hydroxylysine	2.6
Lysine	5.4
Histidine	1.3
Arginine	10.6
Isodesmosine *	5.3
Desmosine *	8.3
Merodesmosine *	0.5
Lysinonorleucine *	2.3

* Expressed as lysine equivalents

Table 7. Amino Acid composition of elastin from bovine ligamentum nuchae treated with collagenase purified by affinity chromatography. (Values are expressed as residues/1000 total amino acid residues and have been corrected for hydrolytic losses).

Hydroxyproline	8.1
Aspartic Acid	5.8
Threonine	9.3.
Serine	8.7
Glutamic Acid	15.5
Proline	116.1
Glycine	329.7
Alanine	228.2
Valine	132.2
Half-cystine	-
Methionine	-
Isoleucine	24.0
Leucine	59.7
Tyrosine	5.9
Phenylalanine	29.3
Hydroxylysine	-
Lysine	3.3
Histidine	0.5
Arginine	5.8.
Isodesmosine*	6.2
Desmosine *	8.9.
Merodesmosine *	0.5
Lysinonorleucine*	2.2

* Expressed as lysine equivalents

Table 9. (75). The amino acid composition of salt-soluble Elastin from the ligamentum nuchae of Copper-deficient calves. (Values are expressed as residues/1000 amino acid residues).

Hydroxyproline	8
Aspartic acid	6
Threonine	8
Serine	9
Glutamic acid	15
Proline	92
Glycine	316
Alanine	220
Valine	147
Cystine	0
Methionine	0
Isoleucine	20
Leucine	55
Tyrosine	6
Phenylalanine	32
Isodesmosine	0
Desmosine	0
Histidine	Trace
Hydroxylysine	0
Lysine	49
Arginine	5

Table 10. The amino acid compositon of Insoluble Elastin isolated from bovine ligamentum nuchae by autoclaving. (Values are expressed as residues/1000 amino acid residues and have been corrected for hydrolytic losses).

Hydroxyproline	8.8
Aspartic Acid	6.5
Threonine	9.2
Serine	9.3
Glutamic Acid	17.5
Proline	115.8
Glycine	317.7
Alanine	226.6
Valine	116.9
Half-cystine	-
Methionine	-
Isoleucine	25.6
Leucine	63.2
Tyrosine	9.0
Phenylalanine	30.4
Hydroxylysine	-
Lysine	3.2
Histidine	0.45
Arginine	6.2
Isodesmosine*	6.9
Desmosine *	10.3
Merodesmosine*	0.7
Lysinonorleucine*	2.3

* Expressed as lysine equivalents

Table 11. The amino acid composition of Insoluble Elastin isolated from bovine ligamentum nuchae by hot alkali treatment. (Values are expressed as residues/1000 amino acid residues and have been corrected for hydrolytic losses).

Hydroxyproline	7.0
Aspartic Acid	5.9
Threonine	6.3
Serine	6.3
Glutamic Acid	15.7
Proline	115.7
Glycine	324.1
Alanine	238.9
Valine	132.0
Half-cystine	-
Methionine	-
Isoleucine	24.9
Leucine	62.0
Tyrosine	7.7
Phenylalanine	30.4
Hydroxylysine	-
Lysine	3.9
Histidine	0.5
Arginine	5.9
Isodesmosine*	6.7
Desmosine *	9.1
Merodesmosine*	nd
Lysinonorleucine*	1.8

* Expressed as lysine equivalents

Table 12. Amino acid composition of elastin from bovine aorta. (Values are expressed as residues/1000 total amino acid residues and have been corrected for hydrolytic losses).

Method of Purification	Enzymic	Enzymic + hot alkali.
Hydroxyproline	10.6	10.1
Aspartic acid	6.5	5.5
Threonine	9.6	6.1
Serine	9.2	6.4
Glutamic Acid	16.0	13.7
Proline	112.7	117.6
Glycine	332.4	337.1
Alanine	223.9	226.0
Valine	131.3	132.4
Half-cystine	0.0	0.0
Methionine	0.0	0.0
Isoleucine	23.5	23.9
Leucine	58.2	57.9
Tyrosine	7.5	6.8
Phenylalanine	29.7	29.5
Hydroxylysine	0.0	0.0
Lysine	4.6	3.9
Histidine	0.5	0.6
Arginine	5.9	4.2
Ornithine	0.0	1.1
Isodesmosine *	5.5	5.5
Desmosine *	9.6	8.7
Merodesmosine *	0.6	0.6
Lysinonorleucine *	2.1	2.5

b). Hexose and Hexosamine Analyses

Analyses for hexose and hexosamine were performed on aliquots of enzymically-purified ligament and aortic elastin sufficiently large to allow the identification of approximately one carbohydrate unit/1000 amino acid residues. Neither neutral hexose nor hexosamine was detected by any of the analytical techniques employed on either aortic or ligament elastin.

c). Amino End-Group Analyses

The following recovery values (71) were used to correct for losses occurring during the regeneration of free amino acids from their dinitropyridyl derivatives.

Table 13

Amino Acid	% Recovery on regeneration in 30% NH_4OH at 100°C for 35 minutes.
∝ - arginine	75
∝ - histidine	88
aspartic acid	84
glutamic acid	84
alanine	90
phenylalanine	80
glycine	80
leucine	85
isoleucine	85
∝ - ϵ - lysine	75
proline	85
serine	86
threonine	85
valine	82

The/

The results of the end group analyses carried out on two elastin preparations isolated from ligamentum nuchae by enzymic treatment are shown in Table 14. Columns b and a report the N-terminal profiles of elastin in which the condensation of 2 - Cl - 3,5 - dinitropyridine and the free amino groups was carried out in the presence or absence, respectively, of dimethyl sulphoxide, which induces a marked swelling of the protein (59,60), and presumeably increases the rate of reaction. The close similarity of these values indicates that a prolonged exposure to 2 - Cl - 3,5 - dinitropyridine even in the absence of a swelling agent ensures an adequate identification of N-terminal residues located in the interior of the macromolecular structure. This view is also supported by the overall agreement of these results with those of the end group estimation by cyanate in the presence of guanidine hydrochloride (column c). However, the identification of a peptide fraction, rich in polar residues and particularly glutamic acid, contaminating the hydantoins after ion-exchange chromatography raises the possibility that some of the free amino acids identified after the regeneration step may not represent true N-terminal residues, even though a good separation of hydantoins from peptides was obtained by gel exclusion chromatography (the elution profile is shown in Fig.6). In view of this limitation it was considered that the 2 - chloro - 3,5 - dinitropyridine method, with its high and uniform sensitivity towards all amino terminal groups, was a more practical procedure for

the/

Fig. 6. The elution profile of Elastin Hydantoins and Peptides on Sephadex G-15.

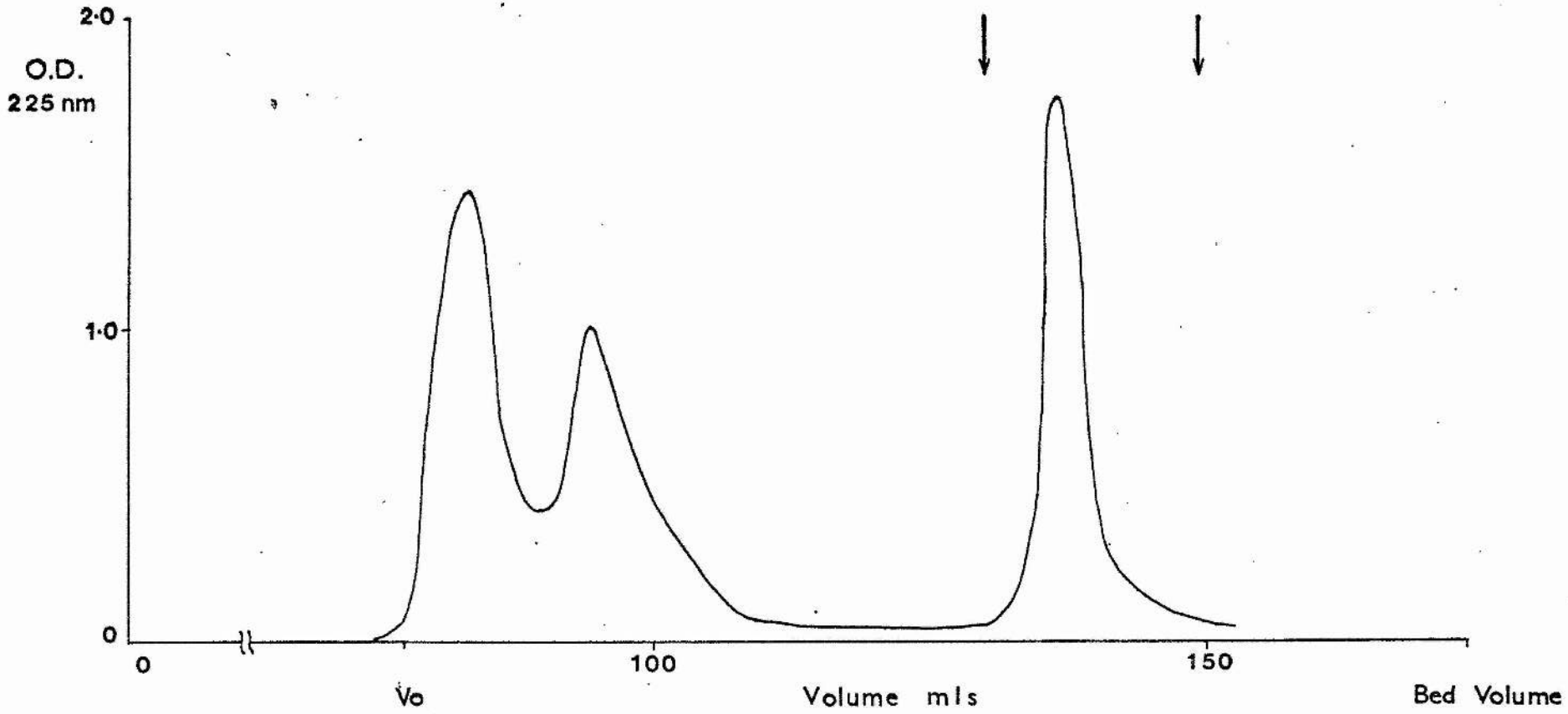
Load: Hydantoins and peptides, from 100 mg elastin, in 3 ml 0.02M NH_4OH

Eluant: 0.02M NH_4OH

Flow rate: 7ml/h

Column size: 1.6 x 95 cm

The peak between the two arrows represents the hydantoins


2·0
O.D.
225 nm
1·0
0
0
100
150
Vo
Volume mls
Bed Volume

the end group analysis of elastin. The N-terminal profile, determined by the chlorodinitropyridine method, of elastin, isolated using collagenase purified by affinity chromatography is reported in column d.

For purposes of comparison Table 15 (18) reports the N-terminal profiles of bovine ligamentum nuchae elastin isolated either by thermal treatment (15) or by a combination of thermal and hot alkali treatments (17).

Table 16 shows the amino end-group profile of aortic elastin isolated using collagenase purified by affinity chromatography, together with that of the same preparation after 45 minutes exposure to boiling NaOH. Before treatment with alkali the preparation revealed only 1.64 moles of N-terminal amino acid residues per 10^6g of protein. However, treatment with hot alkali produced a twenty-two-fold increase in end-group concentration, although the amount of protein solubilised by the treatment was less than 5% of the preparation.

Table 14 N-Terminal Amino Acid Residues of Elastin from Bovine Ligamentum nuchae (Values are expressed as moles of amino acid per 10^6 g of protein. Results are corrected for regeneration losses.)

Method of Purification:	Collagenase purified by chromatography on G-200 and DE 52			Collagenase purified by affinity chromatography
Method of end-group determination:	Dinitropyridine	Dinitropyridine in dimethylsulphoxide	Cyanate	Dinitropyridine
	(a)	(b)	(c)	(d)
Aspartic acid	0.24	0.26	0.15	-
Threonine	-	0.10	0.08	-
Serine	0.15	0.20	0.40	-
Glutamic acid	0.10	0.11	0.21	-
Proline	0.10	0.15	0.39	-
Glycine	1.24	1.21	1.31	0.59
Alanine	0.60	0.87	1.25	0.30
Valine	0.33	0.44	0.56	0.16
Isoleucine	0.49	0.25	-	-
Leucine	-	0.48	0.19	0.08
Tyrosine	0.14	0.07	-	-
Phenylalanine	0.14	0.33	0.04	-
Totals	3.53	4.47	4.58	1.13

Table 15 (18)

N - Terminal amino acid residues of elastin from bovine ligamentum nuchae (values are expressed as moles of amino acid per 10^6g of protein. Results are corrected for regeneration losses.)

Method of Purification	Thermal Treatment	Thermal treatment followed by Alkaline Treatment
Aspartic acid	0.34	0.19
Threonine	0.06	0.12
Serine	0.27	0.25
Glutamic acid	0.05	tr.
Proline	0.20	0.94
Glycine	1.28	8.99
Alanine	0.72	2.80
Valine	0.23	0.96
Isoleucine	0.10	0.18
Leucine	0.34	0.59
Tyrosine	0.06	0.11
Phenylalanine	0.07	0.27
Totals	3.72	15.40

Table 16 N - Terminal Amino acid residues of Elastin from Bovine Aorta.

(Values are expressed as moles of amino acid/10^6g of protein. Results are corrected for regeneration losses).

Treatment	Enzymic	Enzymic + 0.1N NaOH for 45 minutes at 98 °C.
Aspartic Acid		0.56
Threonine		0.34
Serine		0.73
Glutamic Acid		0.36
Proline		4.12
Glycine	0.48	11.01
Alanine	0.47	7.61
Valine	0.36	3.95
Isoleucine		1.86
Leucine	0.23	3.04
Phenylalanine	0.10	3.21
Total	1.64	36.79

d). Estimation of Acetyl Groups

Gas chromatographic analysis of the tert-butylethyl ether extract of a sulphuric acid hydrolysate of ligament elastin (isolated by the use of collagenase purified by affinity chromatography) failed to reveal the presence of acetic, propionic or butyric acid, although an aliquot of elastin sufficiently large to allow the detection of approximately 1.5 acetyl, propionyl or butyryl groups per 1000 amino acid residues was hydrolysed.

Removal of N-Terminal Blocking Groups

The molecular weight of the elastin precursor, tropoelastin, has been reported to range between 30 000 and 100 000 daltons (41, 42); consequently none of the N-terminal residues identified in enzymically purified elastin was present at a sufficiently high concentration to be considered the true end-group of the protein, which must therefore carry a blocked α- amino function.

Attempts were made to remove the blocking group from ligament and aortic elastin (both isolated using collagenase purified by affinity chromatography) using mild alkaline and mild acid treatments.

Table 17 shows the N-terminal profiles of ligament elastin, untreated and after exposure to alkali or acid. Fig. 7 traces the rise in the concentration of N-terminal groups with the time of exposure to 1.5N HCl in methanol (dried with CaO) at 18°C.

The effect of a mild alkaline treatment (0.25N NaOH in dioxan/water for 1 hr at 18°C) upon the N-terminal profile of aortic elastin is shown in Table 18. A marked rise in the concentration of glycine, with little increase in the concentration of other N-terminal residues, was caused by the exposure of aortic elastin to NaOH under these conditions.

Table 17 N - Terminal amino acid residues of Elastin from Bovine Ligamentum nuchae. (Values are expressed as moles of amino acid per 10^6g of protein. Results are corrected for regeneration losses. Values in brackets are molar concentration normalised with respect to Leucine).

Treatment	Untreated	NaOH/Dioxan 0.25N, 60 min 18 °C	NaOH/Dioxan 0.5N, 60 min 18 °C	MeOH/HCl 1.5N 60 min, 26°C H_2O,1000ppm	MeOH/HCl 1.5N 120 min 18°C
Aspartic Acid			1.09	0.77	
Threonine			0.54	0.62	
Serine			2.20		
Glutamic Acid			0.24		
Proline		0.65	0.71		
Glycine	0.59 (7.4)	4.93 (29.0)	10.58 (17.1)	10.04 (9.6)	3.76 (13.0)
Alanine	0.30 (3.8)	0.65 (3.8)	2.69 (4.3)	4.10 (3.9)	1.55 (5.3)
Valine	0.16 (2.0)	0.27 (1.6)	0.69 (1.1)	2.54 (2.4)	1.38 (4.8)
Leucine	0.08 (1.0)	0.17 (1.0)	0.62 (1.0)	1.05 (1.0)	0.29 (1.0)
Phenylalanine		0.08			
Total	1.13	6.75	19.36	19.12	6.98

Fig. 7. The liberation of amino end-groups upon exposure of ligamentum nuchae elastin to 1.5N HCl in Methanol at 18°C. Values are expressed as moles of N-terminal residues per 10^6g of protein, and have been corrected for regeneration losses.

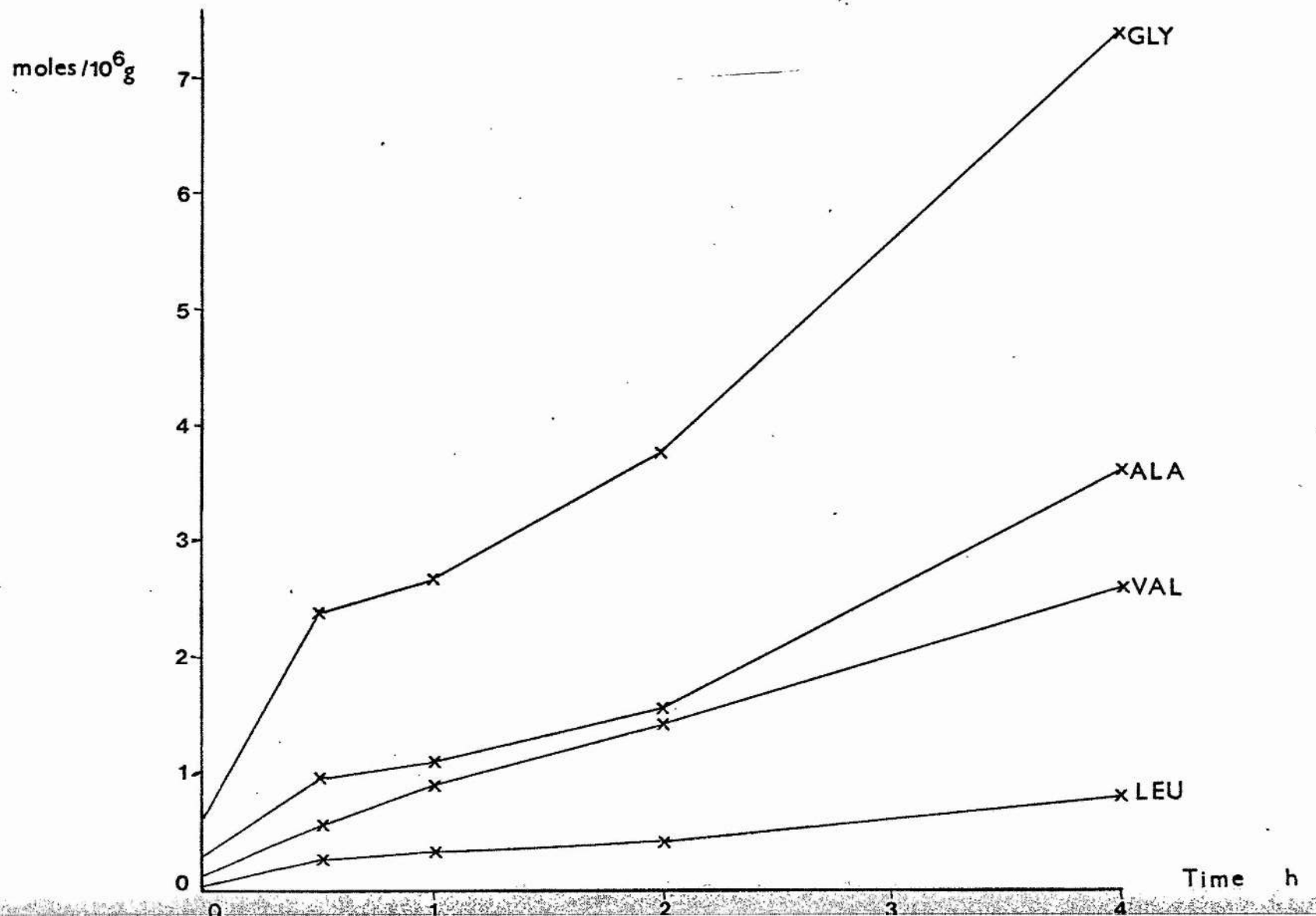

moles /10^6 g
7
6
5
4
3
2
1
0
GLY
ALA
VAL
LEU
Time h
0
1
2
3
4

Table 18 N - Terminal amino acid residues of Elastin from bovine aorta.

(Values are expressed as moles of amino acids/10^6g of protein. Results are corrected for regeneration losses. Values in brackets are molar concentrations normalised with respect to Leucine).

Treatment	Untreated	0.25N NaOH in dioxan for 60 minutes at 18 C
Proline		0.83
Glycine	0.48 (2.1)	7.14 (24.6)
Alanine	0.47 (2.0)	0.82 (2.8)
Valine	0.36 (1.6)	0.41 (1.4)
Leucine	0.23 (1.0)	0.29 (1.0)
Phenylalanine	0.10 (0.4)	0.18 (0.6)
Total	1.64	9.67

Electron Microscopy

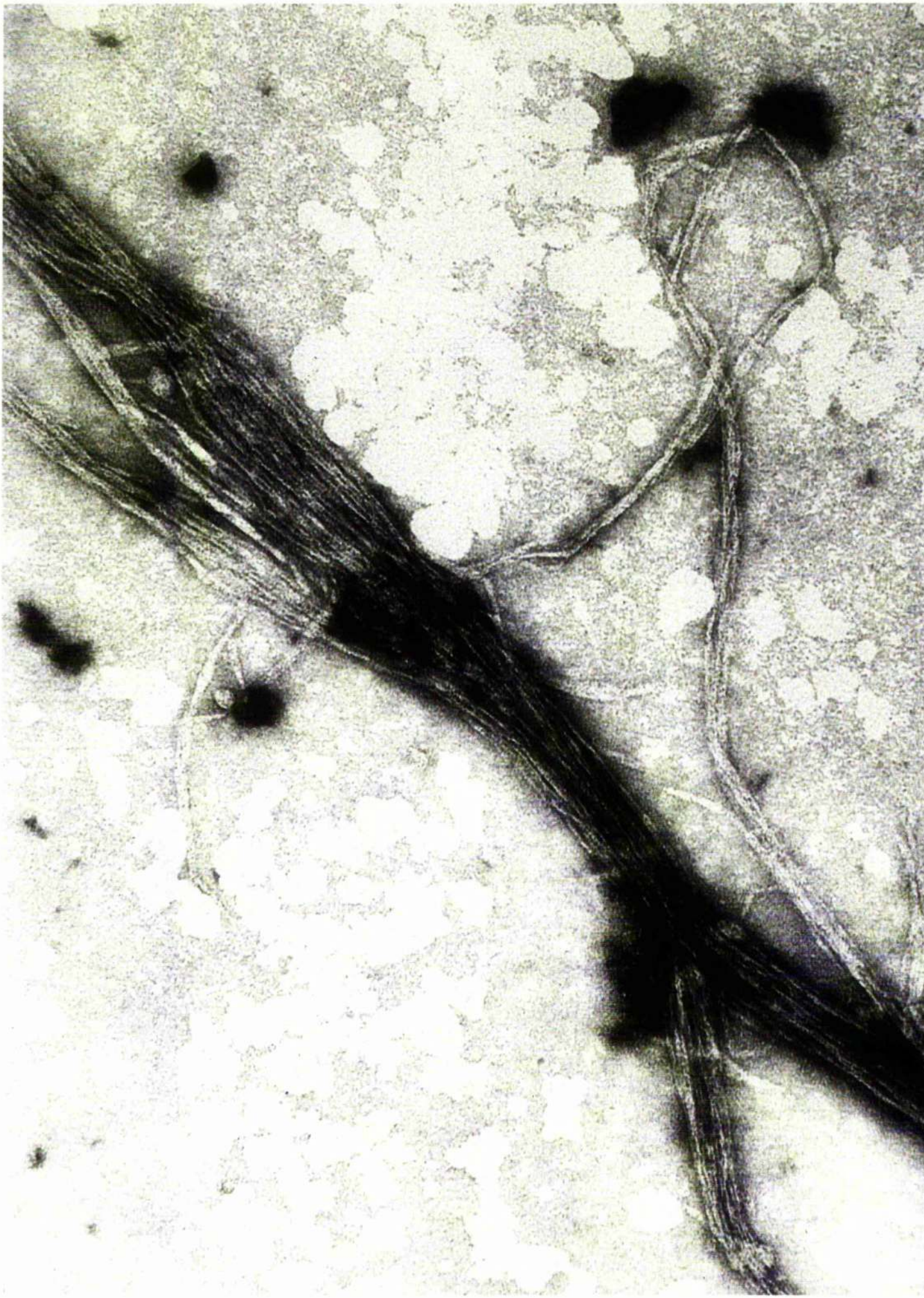
Examination of finely powdered elastin (isolated from ligamentum nuchae by the use of collagenase purified by affinity chromatography) by electronmicroscopy showed that the morphology of the protein was affected by the procedure adopted in the preparation of the specimens. The majority of the fibrils comprising unsonicated samples which had been negatively contrasted in the absence of dimethyl sulphoxide did not reveal any ultrastructural features. Only the smallest fibrils, such as that shown in Fig.8, were resolved into filaments aligned parallel to the major axis of the fibril. However, preparations treated with dimethyl sulphoxide were found to exhibit a lateralarray of filamentous units in all fibrils, irrespective of size (Fig.9 and 10). The filaments appeared 'beaded' in through-focus series of micrographs, with an axial periodicity of 3.9 ± 0.6 nm. The limitation imposed by the staining techniques on the determination of particle dimensions of this size range (76) did not allow an evaluation of the diameter of the filaments, consequently, only the centre to centre distance of adjacent filaments was measured and found to range between 4.5 and 5.3 nm. Prolonged sonication appeared to affect the packing but not the ultrastructure of the filaments within fibrils. In fact, in such preparations individual filaments or small groups of filaments were observed to have become separated from the bulk of fibril, as shown in Fig.11.

Fig. 8. Elastin fibril, negatively stained with uranyl formate - oxalic acid pH 6.4. 142,000 x

Fig. 9. Elastin fibril, negatively stained with uranyl formate - oxalic acid. Penetration of stain was enhanced with dimethylsulphoxide. 269,000 x

Fig. 10. Elastin fibril, negatively stained with uranyl formate - oxalic acid. Penetration of stain was enhanced with dimethylsulphoxide.
648,000 x

Fig. 11. Sonicated elastin fibril, negatively stained with uranyl formate - oxalic acid.
269,000 x



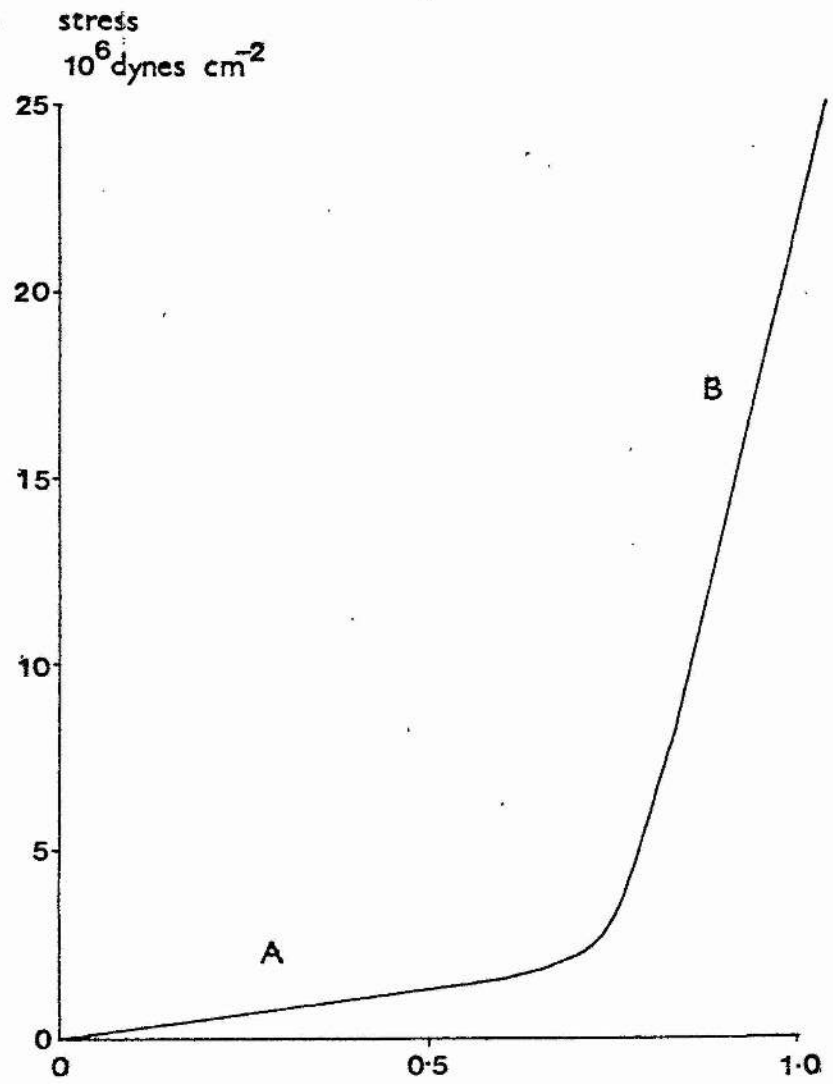
Investigation of Mechanical Properties.

The amino acid composition of the purified elastin strips was found to be identical to that reported earlier for powdered elastin isolated from bovine ligamentum nuchae.

Two typical stress-strain curves obtained with specimens of untreated ligamentum nuchae (I) and purified elastin (II) are shown in Fig.12. Data derived from such curves are presented in Table 19 , and represent average values, each calculated from the stress analysis of ten samples. Under the experimental conditions used in these tests none of the groups of specimens was seen to exhibit hysteresis upon repeated extension - relaxation cycles, provided care was taken in the case of collagenase treated material not to over stress the samples and so cause partial rupture. As stated earlier, marked slippage under repeated stress was observed with strips the ends of which had not been embedded in resin.

Fig. 12. Typical stress-strain curves of samples of untreated bovine ligamentum nuchae (I) and of purified elastin (II).

I


stress
10^6 dynes cm^{-2}
25
20
15
10
5
0
A
B
0
0·5
1·0
Strain

II

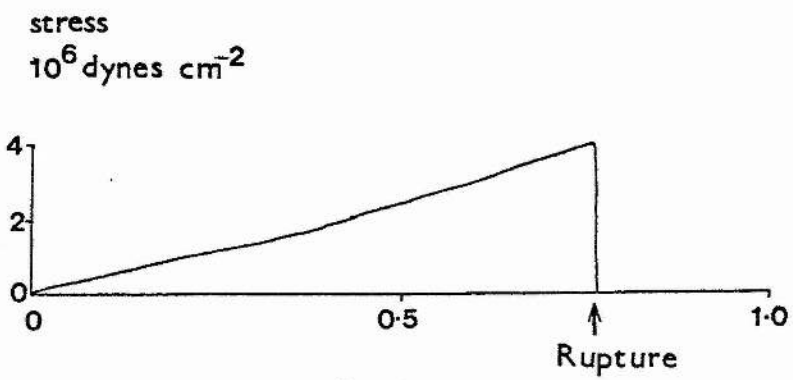

stress
10^6 dynes cm^{-2}
4
2
0
0
0·5
1·0
Rupture
Strain

Table 19. Parameters derived from Dynamometry

	Young's Modules (10^6 dynes cm^{-2})		Stress (10^6 dynes cm^{-2})		Strain	
	Portion of curve		At inflection	At yield	At inflection	At yield
	A	B				
Untreated Ligamentum nuchae	4.7.	153.0	3.3	N.D.	0.72	1.25
Treated only with DTT in 5M GuCl	5.7	35.0	3.6	23.0	0.39	0.98
Treated only with collagenase	5.1	-	-	4.2	-	0.86
Purified Elastin	5.5	-	-	3.9	-	0.77

X-Ray Analysis

X-ray studies were carried out on both orientated and unorientated samples of ligament elastin, isolated using collagenase purified by affinity chromatography.

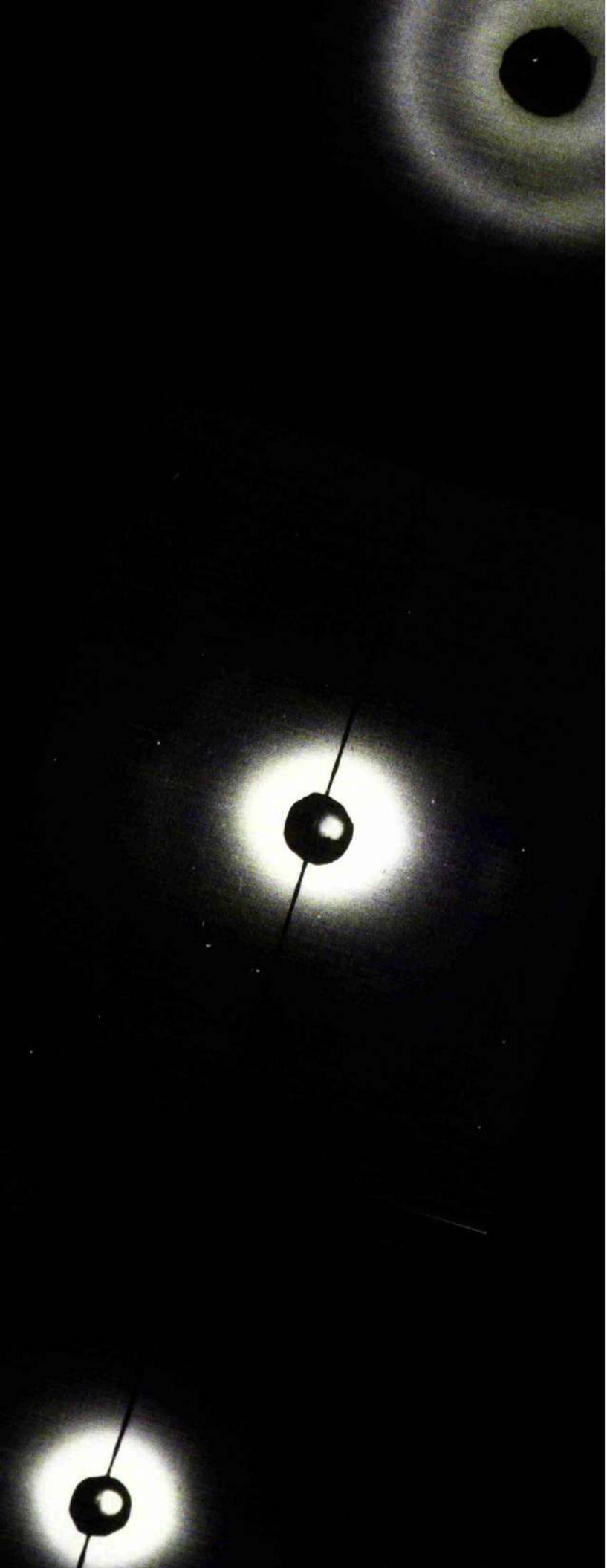
Wide-angle x-ray scattering patterns from the unorientated material showed broad amorphous peaks corresponding to spacings of 9.3 A and 4.5 Å. These features were unchanged in the orientated material. Fig.13 shows a typical wide-angle scattering pattern.

Unorientated samples gave rise to small-angle x-ray scattering patterns in which no significant features are visible. However, samples orientated by extension to 140% of their original length produced a diffuse, almost complete diffraction ring, with a maximum intensity at the equator, corresponding to a spacing of 50 Å (Fig.14). Upon further elongation of specimens to 165% of their original length the 50 Å reflection becomes almost entirely equatorial and is accompanied by an additional equatorial reflection, corresponding to a spacing of 46 Å. The small-angle x-ray scattering pattern of a sample at an elongation of 165% is shown in Fig.15.

Fig. 13. Wide-angle x-ray diffraction pattern of dry elastin from bovine ligamentum nuchae.

Fig. 14. Small-angle x-ray diffraction pattern of elastin from bovine ligamentum nuchae. Sample was stretched to 140% of its original length and dried.

Fig. 15. Small-angle x-ray diffraction pattern of elastin from bovine ligamentum nuchae. Sample was stretched to 165% of its original length and dried.

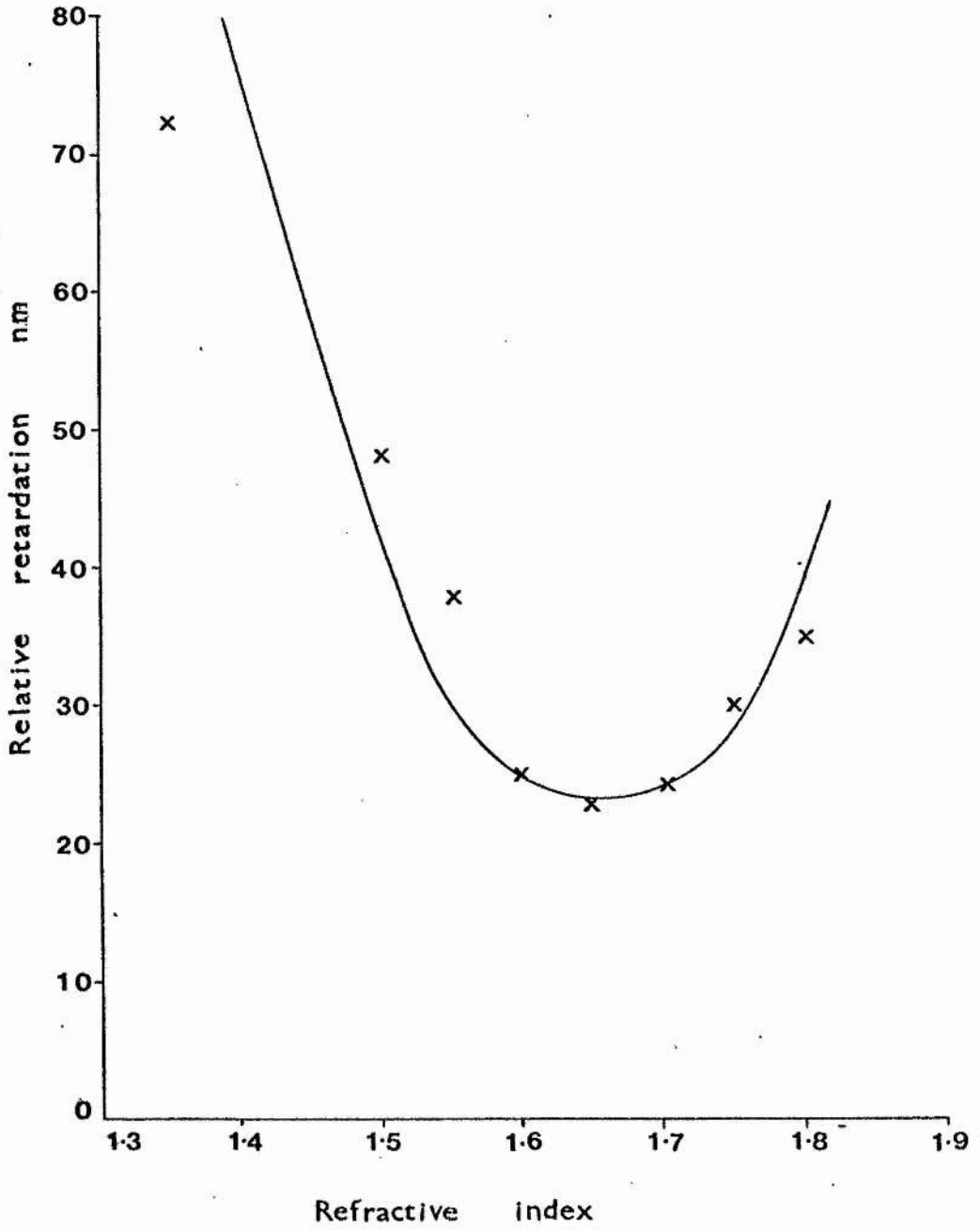


Optical Polarisation Analysis

When longitudinal and transverse sections of elastin strips were examined under the polarizing microscope, it was apparent that the fibres exhibited positive uniaxial birefringence, in common with most biological fibres. Only a few of the many fibres comprising of longitudinal section were in the correct angular relationship to the polariser at any one orientation of the sample.

The variation in the relative retardation of individual fibres on changing the refractive index of the medium is illustrated in Fig. 16. The shape of the curve indicates that the elastin fibres possessed both form and crystalline birefringence. In water the value of the birefringence of elastin, estimated from measurements of the relative retardation of individual fibres, was approximately 1×10^{-2}.

Fig. 16. Variation in the relative retardation of elastin fibres with the refractive index of the surrounding medium.


80
70
60
50
40
30
20
10
0
Relative retardation nm
1·3
1·4
1·5
1·6
1·7
1·8
1·9
Refractive index

DISCUSSION

In this study a slightly modified version of the isolation procedure of Ross and Bornstein (22) was adopted in the purification of elastin from two sources, namely bovine ligamentum nuchae and aorta.

The elastin from ligamentum nuchae has been studied extensively by numerous workers (1,2,15,22) and its composition characterised. The amino acid profile of ligamentum nuchae elastin purified in this investigation by the use of a collagenase preparation free of detectable elastolytic activity (Table 7) is virtually identical to that of the protein sample isolated from the same tissue by treatment with hot alkali (Table 11), the only significant difference being the higher content of serine and threonine in the former preparation. Similar compositional differences have been observed between elastin preparations purified by thermal (Table 10) and alkaline treatment and have been attributed to contaminants not removed by autoclaving (2,6). One such substance, isolated from thermally-purified ox ligamentum nuchae elastin, has been identified as a glycoprotein (28,29). However, the presence of a similar contaminating material in enzymically purified elastin from ligamentum nuchae is ruled out by the failure to detect either hexosamines or neutral sugars in samples sufficiently large to allow the identification of these substances at a level of approximately one unit per 1 000 amino acid residues. Furthermore, the concentrations of serine and threonine in tropoelastin (75,77) (Table 9)

are/

are greater than in NaOH - purified elastin and are comparable to those in enzymically - purified preparations (Table 7), an observation incompatible with the presence of contaminating glycoproteins in the enzymically purified material since tropoelastin contains neither neutral sugars nor hexosamine (77). The presence of any appreciable amount of collagen in the elastin isolated by enzymic treatment is excluded on similar grounds, for hydroxyproline is present in salt-soluble elastin isolated from the ligamentum nuchae of copper-deficient calves at a concentration of approximately 8 residues per 1000 amino acid residues, compared to 8.1 residues per 1000 amino acid residues reported in this investigation for enzymically-purified elastin.

Previous end-group analyses conducted on sodium hydroxide purified elastin from ligamentum nuchae have revealed the presence of several amino acid residues in terminal positions (1,2,16). However, the concentrations of these residues were lower than the values reported by Spina and Fracassini (Table 15) (18). The procedure adopted in the latter case for the determination of amino end-groups was the chlorodinitropyridine method (69) rather than the fluorodinitrobenzene method of Sanger (78) as used by the other investigators. The latter technique does not appear to be as suitable as the chlorodinitropyridine method for use with elastin, since some destruction of the DNP-amino acids, particularly DNP-glycine, occurs on hydrolysis of the DNP-protein (79). As such losses vary according

to/

to the protein in question (80) it is difficult to make corrections for them. The advantages of the chlorodinitropyridine procedure are the uniform and high recoveries (of the order of 80 - 90%) of all amino acids (Table 13) (70).

The amino end-group contents of the enzymically purified elastin preparations from ligamentum nuchae are far lower than that of the elastin preparation purified by both thermal and alkaline treatment (Table 15) (3.53 and 1.13 moles of N-terminal per 10^6g protein for the enzymically purified preparations as opposed to 15.4 moles N-terminal per 10^6g protein for elastin purified by thermal and alkaline treatment). The effectiveness of affinity chromatography in removing elastolytic proteases from the collagenase preparation is evident when comparison is made of the N-terminal profile of elastin obtained using the collagenase preparation purified by gel exclusion and ion-exchange chromatography (Table 14, column a) with that of the protein isolated by the use of collagenase purified by affinity chromatography (Table 14, column d). Not only is there an overall decrease in the molar concentration of N-terminal residues (from 3.53 to 1.13 moles N-terminal per 10^6g protein), there is also a reduction in the number of amino acid residues contributing to this total. At the low level of free amino groups detected in elastin isolated from ligamentum nuchae by the use of collagenase purified by affinity chromatography, it is open to speculation

whether/

whether they result from peptide bond cleavage which had occurred during the isolation procedure or reflect damage incurred in vivo as a result of the extremely low turnover of the protein.

To date, treatment with hot alkali (17) has been considered to be the only reliable method available for the purification of elastin from contaminating macromolecules in adult bovine aorta (2). In the present study the protein has been isolated from thistissue by the use of collagenase, purified by affinity chromatography, in conjunction with guanidine and dithiothreitol. Comparison of the amino acid composition of enzymically purified aortic elastin with that of the same preparation following exposure to hot alkali clearly reveals that treatment with NaOH results in a significant reduction in the concentration of some polar amino acid residues, particularly serine and threonine (Table 12). As already mentioned, similar compositional differences between thermally and alkali treated elastin preparations from ligamentum nuchae have been attributed to contaminating glycoproteins. However, as in the case of enzymically purified elastin from ligamentum nuchae, neither hexosamine nor neutral sugar were detected in aortic elastin isolated by the use of collagenase, ruling out the possibility of contaminants

of/

of this nature. Equally incompatible with the presence of any substantial amount of contaminant is the extremely low concentration of N-terminal residues identified in the enzymically purified preparation (Table 16). In keeping with this is the observation that treatment of a collagenase purified sample with hot alkali resulted in less than a 5% loss of weight from the elastin, as compared with the solubilisation of about 17% of the preparation when autoclaved aortic elastin was similarly treated with alkali (6). In the present case, 25% of this loss is accounted for by the decrease in concentration, in the residue, of only four amino acids, namely aspartic acid, glutamic acid, threonine and serine. However, the N-terminal analysis of the sample subjected to hot-alkali treatment revealed a twenty-two-fold increase in end-group concentration, indicative of extensive cleavage of the elastin polypeptide backbone. It is worth noting that the total amount of N-terminal residues detected in the hot-alkali treated elastin is one order of magnitude greater than that observed for a similar preparation by Gotte et al. (2), but is in keeping with the values published for human aorta by Taylor (81). These results strongly indicate that compositional differences between enzymically and alkali treated preparations arise from the loss of short peptides of elastin caused by hydrolytic damage rather than from the solubilisation of contaminating substances.

The/

The amino acid profiles of enzymically prepared elastin from bovine aorta and ligamentum nuchae (Tables 7 and 12) appear to be identical within the limits of experimental accuracy with the exception of the concentrations of three residues, namely hydroxyproline, lysine and tyrosine. It is open to speculation whether the small differences observed in the levels of hydroxyproline and lysine in the two preparations may reflect slightly different degrees of activity of proline hydroxylase and lysyl oxidase in the two tissues.

It would appear, therefore, that the use of collagenase, purified by affinity chromatography, in conjunction with guanidine and dithiothreitol results in the isolation of pure insoluble elastin from both bovine aorta and ligamentum nuchae. That this mild procedure yields undegraded samples of the protein is indicated by the low level of N-terminal groups detected in the preparations - 1.13 moles per 10^6g of protein in the case of ligament elastin, 1.64 moles per 10^6g of protein in elastin from aorta.

In both aortic and ligament elastin, isolated by the use of collagenase purified by affinity chromatography, the amino acid residue present in the highest concentration as an amino end-group is glycine, occurring to the extent of 0.48 moles per 10^6g of protein in aortic elastin and 0.59 moles per 10^6g protein in elastin from ligamentum nuchae. Such low levels of N-terminal are clearly incompatible with the molecular weight of the elastin precursor, isolated from copper-deficient animals, which has been estimated to be 74,000 daltons (43). This discrepancy can be interpreted in two ways. The free N-terminal of the precursor (which has been identified as glycine (44)) may undergo, during elastin maturation, a chemical reaction rendering it undetectable under the experimental conditions used in N-terminal analysis. The other possibility is that it is buried in the interior ofthe macromolecule and inaccessible to the reagent. This second explanation is ruled out by the observation that the N-terminal profile of elastin from ligamentum nuchae is independent of the degree of swelling of the protein during analysis (Table 14).

In an attempt to determine the nature ofthe true N-terminal residue of insoluble elastin the protein was subjected to mild acid or alkaline treatments. It was hoped that in this way the group blocking the amino function of the N-terminal residue would be removed without a rise in the general background level of amino

end-groups/

end-groups so large as to obscure the presence of the unblocked residue.

Treatment with methanolic HCl of elastin from ligamentum nuchae did not appear to selectively liberate any particular residue as an N-terminal (Table 17 and Fig.7), an observation which rules out the possiblity of the amino function being blocked by a formyl group, since this groups is rapidly removed under mild acid conditions (82). Furthermore, the failure of gas chromatographic analysis to detect acetic, propionic or butyric acid in sulphuric acid hydrolysates of elastin from ligamentum nuchae eliminates the possiblity of the N-terminal residue carrying an acetyl, propionyl or butyryl group.

Exposure to sodium hydroxide in dioxan of aortic and ligament elastin resulted in both cases in a large increase in the concentration of N-terminal glycine with only a slight accompanying rise in the concentration of other N-terminal residues. These results may only indicate the presence in elastin of particularly labile peptide bonds involving glycine. On the other hand they could arise from the base-catalysed removal of a function which masks the amino group of N-terminal glycine in mature elastin. In this respect it is worthy of note that this amino acid has been identified as the N-terminal residue of tropoelastin isolated from the aorta of copper-deficient swine, as already mentioned (44). However, in neither aortic nor ligament elastin subjected to mild alkaline

treatment/

treatment does the concentration of glycine as amino end-groups reach a level sufficiently high as to be compatible with the molecular weight of tropoelastin.

The following, although of a highly speculative nature, might account for the above observations. The first step postulated by Partridge and associates in the biosynthesis of elastin cross-links was the oxidative deamination of lysyl residues in the polypeptide chains to form residues of the δ-semialdehyde of α-aminoadipic acid (allysine) (83,84), later identified in purified elastin preparations (12,85,86). Residues of allysine may condense with one another and/or with the ϵ-amino group of unmodified lysyl residues to form both desmosine and lysinonorleucine (87,- 91). It is suggested that the free amino function of N-terminal glycine condenses with an allysyl residue to form a Schiff base, in the same way that a lysyl side chain may react with an allysyl residue to form the Schiff base $\Delta^{6,7}$ - dehydrolysinonorleucine (86). It is possible that the Schiff base formed from N-terminal glycine and an allysyl residue might be very labile to cleavage under mild alkaline conditions but show some degree of stability in anhydrous methanolic HCl, when a methanol adduct might be formed. It would be expected that , under physiological conditions a proportion of the Schiff bases would be reduced, resulting in

stable

stable derivatives such that the amino function of N-terminal glycine would not be liberated upon treatment with alkali. If this situation did exist the concentration of N-terminal glycine detected after mild alkaline treatment would be less than the 13.5 moles per 10^6g of protein necessary to account for the molecular weight of tropoelastin.

The electron microscopic evidence presented in this study essentially corroborated previous observations on the ultrastructure of elastin conducted on preparations of bovine ligamentum nuchae and human aorta purified by alkali and/or thermal treatment (50,34). It demonstrates that the fibrils consist of a parallel array of primary filaments of indefinite length. The presence of such filaments in samples not subjected to sonication or exposed to dimethylsulphoxide rules out the possibility of this ultrastrudtural pattern arising from conformational changes induced by these treatments.

It should be mentioned that the identification of primary filaments in elastin has been attributed, on the grounds of a gross morphological similarity, to the presence in the preparation of partly degraded collagen fibrils (51). However, such a claim is not in keeping with the absence of a 2.86 Å meridional and a 10.4 Å equatorial

reflection/

reflection in the wide-angle X-ray scattering pattern reported in Fig.13 (92). Moreover, the collagen fibrils of bovine ligamentum nuchae exhibit an average diameter of about 500 Å which is largely exceeded by that of the elastin fibrils resolved into constituent filaments by either sonication (50) or treatment with dimethyl-sulphoxide (Figs. 9,10).

It is open to speculation whether the remarkable degree of preferential orientation of the primary filaments along the axis of the fibril observed under the electron microscope is typical of the material in the relaxed, hydrated state or has been partly caused by ultrastructural rearrangements induced by vapour - or osmotic-pressure gradients and surface tension forces occurring during the drying-down phase in the preparation of specimens for electron microscopic examination. However, the anisotropy of enzymically purified elastin is supported by the finding that the protein exhibits uniaxial positive birefringence and by the results of the X-ray diffraction analysis. In the interpretation of this evidence, it should be noted that both low-power and scanning electron microscopy have shown the ligament to be composed of interlaced fibres (50). These fibres, which consist of bundles of fibrils, are preferentially but not perfectly orientated relative to the major axis of the ligaments, a fact that may account for the observation that the birefringence of the protein estimated on fibres,

averaging/

averaging 7μm in diameter, exceeds by two order of magnitude the value determined by Gotte et al. with macroscopic samples (59). A birefringence of the order of 1×10^{-2}, as reported in the present investigation, is in the range of values exhibited by proteins with an appreciable degree of structural organisation (93). The lack of alignment of the fibrils in unstretched elastin may also be responsible for the absence of any significant reflection in the small angle X-ray scattering pattern of relaxed specimens. This interpretation is supported by the appearance of a faint and almost complete diffraction ring, with an equatorial intensity maximum (Fig.14),when the coherence of the structural units is enhanced by moderate stretching and by the fact that the reflection becomes almost entirely equatorial upon further elongation of the specimen (Fig.15). This equatorial arc, corresponding to a spacing of 50 Å, is conceived to represent the centre to centre distance of the primary filaments since this value is in good agreement with that obtained from measurements conducted upon electron micrographs (Fig.10). The presence of an additional equatorial reflection at 46 Å in specimens examined at an elongation of 1.65 is suggestive of an uneven distribution of stress among fibrils preceeding the macroscopic failure of the material. This reduced spacing corresponds to the minimum value for the filament centre to centre distance observed in electron micrographs.

The/

The elongation at breakage of samples of enzymically purified elastin is greater than that exhibited by alkali-treated preparations and comparable to that observed with autoclaved elastin (52). This variability may be a reflection of the extent of chain damage induced by the isolation procedure. In this respect it is significant that thermally- and alkali-purified samples exhibit concentrations of N-terminal residues which are respectively 3 and 14 times as high as those found in preparation treated with collagenase. The presence in enzymically purified elastin of only 1 mole of amino end-groups per 10^6g of protein corresponds to one peptide bond cleavage every 300 inter-crosslinkage chain segments, assuming a Mc of 3,400 daltons. This value which has been calculated from the number of lysyl residues utilised in the formation of cross-links, assuming that all such cross-links are inter-chain and unite two chains only (94), is in keeping with that of 3,250 determined from stress-strain measurements (59). This level of damage is so insignificant that it cannot account for the very low tensile strength (3.9×10^6 dynes cm^{-2}) and breaking strain (0.77) of the material which must therefore possess only a minimal degree of structural stablisation and it is probable that macrofibres rupture when the applied stress overcomes the lateral cohesive forces operative at fibre level. It is, however, worth noting that there appears to be

a/

a lack of lateral cohesion even between primary filaments as they can be separated, without apparent damage, by sonication (Fig.11). This finding rules out the possibility of extensive inter-filament cross-linking of a covalent nature interlocking the structures in a three-dimensional network.

Sequence analyses, carried out on the soluble elastin precursor isolated from porcine aortic tissue, have shown that cross-linking of elastin polypeptide chains arises from the interaction of modified lysyl residues located in groups of two or more in alanine-rich sequences which are separated by 35 - 100 amino acid residues (94, 95). Furthermore, at present it appears that even the polyfunctional amino acid desmosine and isodesomsine, which are potentially capable of uniting up to four polypeptide chains, do, in fact, operate only as bifunctional cross-links (96). In view of the high efficiency exhibited in the condensation of oxidised lysyl residues during cross-link formation in elastin, as indicated by the presence in the mature protein of only 5 - 8% of the aminoadipic semialdehyde residues originally available, it is postulated that the primary filament is constituted by several polypeptide chains in lateral alignment, registered in such a way as to maximise the overlap of their cross-linking sites. The presence of only two diffuse rings in the wide-angle X-ray diffraction pattern of both relaxed and stretched elastin and the lack of any meridional reflection in the small-angle X-ray

scattering/

scattering patterns suggest that the segments of polypeptide chains located between adjacent cross-link sites adopt a disordered conformation. This is also indicated by the results of circular dichroism (53), C^{13} nuclear magnetic resonance (97) and Raman Spectroscopy (98) investigations conducted on the intact protein. However, it cannot be excluded that short sequences may exhibit a secondary conformation, possibly in the form of B-turns which have been postulated to occur in some elastin peptides (99,100) and which are characteristic of synthetic polymers of a five-residue repeat (Val-Pro-Gly-Val-Gly) typical of elastin (101.).

If the covalent cross-links are indeed confined within the primary filaments, it is possible that permanent displacement of these structural units relative to one another under stress is prevented by hydrophobic interactions operative in a fashion similar to that proposed by Urry et al. to account for the formation of filaments in coacervates of tropoelastin, α - elastin and synthetic polypentapeptides (102-104). This view is supported by the marked effect of dimethylsulphoxide in enhancing the diffusion of uranyl formate into the interior of the fibril. There is, however, some electron microscopic evidence pointing to a possible participation of covalent cross-links in the stabilisation of elastin fibrils in aorta (34).

Although macroscopic specimens of elastin consist of a net-

work/

work of imperfectly aligned fibres, the evidence presented in this study shows that the material exhibits a high degree of anisotropy at the molecular level in that the fibrils are constituted by axially orientated primary filaments. One, therefore, questions the validity of the application of equations derived for isotropic systems (55,57) to the interpretation of the elastic properties of this fibrous protein.

In contrast to pure elastin, samples of ligamentum nuchae exhibit a high tensile strength (greater than 3×10^7 dynes cm^{-2}) and do not undergo failure before elongations of the order of 1.2. The stress-strain curve of untreated ligament is markedly biphasic, the inflection point occurring at an elongation of approximately 0.72 (Fig. 12,I and Table 19). The first portion of the curve is remarkably linear, the Young's modulus of the material being 4.7×10^6 dynes cm^{-2}, a similar value to that shown by pure elastin (5.5×10^6 dynes cm^{-2}) which, however, undergoes failure above elongations of 0.77. As previously discussed rupture of specimens of elastin would seem to occur because of slippage, which is prevented in the ligament by the inter-relation of elastin and other structural components, most obviously the collagen fibrils which envelope each elastin fibre. The role of the microfibrillar component, which is

presumeably/

presumeably implicated in the behaviour of ligament, is hard to ascertain; treatment of ligamentum nuchae with dithiothreitol results in a system showing a biphasic stress-strain curve, the second portion of which is only one fifth as steep as that for untreated ligament. However, dithiothreitol and guanidine not only solubilise the microfibrillar component but also denature and remove a considerable amount of collagen. When samples of ligamentum nuchae are treated only with collagenase they exhibit a behaviour similar to that of purified elastin, although showing a slightly greater breaking strain. This latter fact may be a consequence of the incomplete removal of collagen since associated mucoprotein, solubilised in guanidine and dithiothreitol in the preparation of pure elastin, may hinder the digestion of collagen by collagenase (21).

SECTION B

MICROFIBRILLAR COMPONENT

INTRODUCTION

Elastin fibres, prepared for electronmicroscopy by routine procedures, have been shown to consist of two morphologically different components: a centrally located, apparently amorphous structure composed of elastin, surrounded by a layer of tubular microfibrils, approximately 11 nm in diameter (105 - 107). These microfibrils, referred to as the microfibrillar component, have been observed in association with elastin in mature elastic fibres from foetal and new-born calf ligamentum nuchae (105 - 108), developing rat tendon (109), and various other tissues. The microfibrillar component shows a marked affinity for lead, osmium and uranyl acetate, in contrast to the amorphous component which stains with phosphotungstic acid (22). Microfibrillar material is not solubilised by autoclaving, but is susceptible to attack by chymotrypsin (EC 3.4.4.5) and is degraded by treatment with hot alkali (110).

The only available reports on thechemical composition of the microfibrillar component are those published by Ross and Bornstein (22,110) who isolated the protein from foetal bovine ligamentum nuchae, rendered collagen-free by exposure to collagenase. The component was solubilised by the reductive cleavage of disulphide bonds with dithioerythritol in 5M guanidine. The amino acid compositions determined for the microfibrillar component in these two investigations are presented in Table 20. The protein is rich

in/

Table 20 The Amino acid composition of the Microfibrillar component isolated from Foetal bovine ligamentum nuchae. (Values are expressed as residues per 1000 amino acid residues).

Reference:	(22)	(110)
Hydroxyproline	n.d.	
Aspartic acid	114.0	127.0
Threonine	55.1	65.0
Serine	58.9	77.5
Glutamic acid	111.0	136.0
Proline	70.4	60.5
Glycine	120.0	106.0
Alanine	58.9	63.0
½ Cystine	60.3	24.8
Valine	54.1	60.5
Methionine	15.8	13.5
Isoleucine	45.2	51.6
Leucine	57.2	74.2
Tyrosine	30.0	33.7
Phenylalanine	32.1	39.3
Lysine	36.9	39.3
Histidine	14.2	10.2
Arginine	45.2	54.0
Tryptophan	n.d.	24.8

in polar and sulphur-containing amino acids and does not contain any of the polyfunctional amino acids characteristic of elastin. In the preparations the protein was found to be associated with both hexose and hexosamine.

The microfibrils may be of considerable importance in the morphogenesis of elastic tissue as several investigators have shown that the earliest recognisable elastic fibres consist solely of bundles of these structures, elastin appearing at a later stage in the development of the fibre (106,107,109,111,112).

An investigation, not here reported, on the structural arrangement of the components of elastic tissue, primarily ligament, is at present in progress in this laboratory. Electronmicroscopic examination of transverse sections, cut serially from rabbit ligamentum flavum, has revealed that there is no insertion of elastin into bone; rather the elastin fibres appear to terminate in a highly collagenous region connected to the vertebra.

As would be expected, removal, by collagenase digestion, of the collagenous component drastically alters the mechanical behaviour of the ligament.

Such/

Such mechanical and electronmicroscopic investigations of ligamentum flavum indicate that its elastic behaviour is the result of a highly organised structural relationship between the various components most obviously elastin and collagen. An understanding of this complex structural relationship would be facilitated by a knowledge of the physical and chemical properties of all the components present in the tissue and it was felt, therefore, that a characterisation of the microfibrillar component, for which there is such a scarcity of data, might prove to be of some interest and value.

MATERIALS AND METHODS

Purification of Collagenase

A commercially available Clostriduim histolyticum collagenase (EC 3.4.4.19) preperation (Sigma (London) Chemical Co., Ltd., Type 1) was batch-purified with DEAE cellulose as described in Section A . The enzyme preparation was then purified further by affinity chromatography, under experimental conditions very similar to those employed in the purification of collagenase to be used in the isolation of elastin. However, the affinity support did not consist of NaOH-treated elastin from bovine ligamentum nuchae (17), but rather of insoluble elastin isolated from bovine ligamentum nuchae by repeated autoclaving (15), as already detailed in Section A. Although autoclaving removes most of the collagen normally associated with elastin it does not solubilise the microfibrillar component (28,29). A column consisting of elastin purified by autoclaving should, therefore, retain those proteins possessing activity towards either elastin or the microfibrillar component when an impure collagenase preparation is passed through it.

An aliquot of the partly purified collagenase preperation was dissolved in 5 ml 15mM Tris (pH 7.5) containing 1 mM $CaCl_2$, and applied, under gravity, to a column (1.3 x 6.0 cm) of coarsely milled elastin - purified by autoclaving - equilibrated with the same buffer. Elution was performed at a flow rate of 20 ml/h at 4°C, the first 18 ml of effluent being collected and stored at 4°C.

Before/

Before use, the enzyme solution was tested for activity towards insoluble collagen (calf-skin) as previously described.

The Isolation of the Microfibrillar Component From Bovine Ligamentum Nuchae.

The microfibrillar component was extracted from ligamentum nuchae by treatment of thetissue with dithiothreitol, which reductively cleaves disulphide bonds. The reformation of these bonds upon removal of the reagent was prevented by reaction of sulphydryl groups with iodoacetic acid, as described by Ross and Bornstein (22), to form a carboxymethylated derivative of the microfibrillar component.

The ligament was removed from the carcasses of three-year old cattle, dissected free from adhering tissues and homogenised in ice-cold H_2O before being defatted and dehydrated in chloroform-methanol (3:1 by volume) for 24 hrs at 4°C. The defatted material was collected, air-dried and ground in a hammer-mill to a fine cream-coloured powder which was extracted with a large volume of 1% NaCl for 24hrs at 4°C, in the presence of a small amount of toluene.

The residue was collected by centrifugation and washed thoroughly with water prior to lyophilisation.

An aliquot (100g) of the lyophilised material was suspended in 1000 ml 5M guanidine hydrochloride (62)-0.1M Tris (pH 7.4) and extracted with continuous stirring for 24 hrs at 4°C. The extraction was repeated for a total of six 24 - hr periods, after which the extraction residue was suspended in 500 ml 5M guanidine hydrochloride - 0.1M Tris (pH 7.4) containing 0.4% EDTA and degassed under reduced pressure before being placed under a nitrogen barrier. Dithiothreitol was added to a concentration of 0.05M, the vessel sealed, and the suspension stirred for 48 hrs at 37°C. At the end of this time the suspension was transferred, under a nitrogen barrier, to an air-tight centrifuge pot and centrifuged at 23 000 x g for 30 minutes. The supernatant was transferred to a light-proof vessel and the pH of the solution adjusted to a value of 8.6 with 1N NaOH. A freshly prepared solution of iodoacetic acid (18.6g, a four-fold molar excess over dithiothreitol) in 1N NaOH (69.6 ml) was then added, the vessel sealed and the reaction mixture stirred for 45 minutes. Light was excluded from the reaction in order to prevent the formation of iodine which may react with histidine and tyrosine residues. A five-fold molar excess of mercaptoethanol over iodoacetic acid was added and the solution dialysed against frequent changes of H_2O for 48 hrs at 4°C, when a white precipitate formed within the dialysis

sac./

sac. Three volumes of cold ethanol were added to the retentate to aid the collection of the precipitate by low speed centrifugation. The precipitate was washed with 50% aqueous ethanol, dehydrated with ethanol, acetone and diethyl ether and air-dried to a fine white powder.

Contaminating collagen was removed by treatment with collagenase purified by affinity chromatography. Aliquots of the dried preparation of S - carboxymethyl microfibrillar component were suspended in 0.01M $CaCl_2$, the pH of the suspension adjusted to a value of 7.5 with 0.01N NaOH and collagenase added to give a residue to enzyme ratio of approximately 500:1. The digestion was allowed to proceed at 37°C and pH 7.5, this value being maintained by the addition of 0.01N NaOH using a Radiometer pH state arrangement, as detailed for the purification of elastin. When there was no indication of further digestion, the suspension was centrifuged at 38 000 x g for 90 minutes, the residue resuspended in 0.01M $CaCl_2$ and the treatment with collagenase repeated. The S - carboxymethyl microfibrillar component was collected by centrifugation and washed with water. Preliminary investigations in which the electron-microscopic appearance of non-alkylated, collagenase treated microfibrillar material was examined indicated that the preparation was contaminated by a substantial amount of very finely divided elastin. In order to remove any such contaminant, the washed alkylated material

was/

Figure 17 Preparation Procedure for S-Carboxymethyl MicroFibrillar component.

Finely milled, defatted bovine ligamentum nuchae

1. 1. 1% NaCl, 24h, 4°C

2. 5M guanidine - 0.1M TRIS pH7.4, 6 x 24H, 4°C

3. 5M guanidine - 0.1M TRIS pH7.4, 0.4% EDTA, 50 mM DTT, 48h, 37°C

4. Centrifugation, 23000 g

5. Supernatant carboxymethylated using I CH_2COOH

6. Dialysis against H_2O, 4°C

7. Addition of ethanol, collection of precipitate

8. Residue treated (2x) with purified collagenase

9. Washed residue dissolved in 5M guanidine hydrochloride (pH7.4)

10. High speed centrifugation (50 000 rpm) 2h.

11. Dialysis against H_2O, 4°C

12. Precipitate collected, washed and dried.

S - Carboxymethyl Microfibrillar component.

was dissolved in 5M guanidine hydrochloride - 0.1M Tris (pH 7.4) and the solution subjected to high speed centrifugation (240 000 x g) for 2 hrs, after which a perfectly clear supernatant was obtained. The S - carboxymethyl microfibrillar component was recovered after dialysis of the solution against H_2O, when a precipitate formed which was easily collected by low speed centrifugation after the addition of three volumes of ethanol. The residue was dehydrated and air-dried.

CHEMICAL ANALYSES

a). Amino acid Analyses

Samples were hydrolysed and amino acids subsequently identified and quantitated as described in Section A.

Hydroxyproline was estimated independently by a colorimetric procedure (63).

Tryptophan was assayed by the modified procedure of Opienska-Blauth et al, a technique based on the colour reaction obtained with glyoxylic acid and the indole ring (113). A solution of D,L - Tryptophan (Sigma (London) Chemical Co., Ltd.) of known concentration

was/

was used as a standard.

b). Hexosamine Estimation

Aliquots (5 mg) of the protein were hydrolysed in 4N HCl under nitrogen for 8 hrs at 110°C. The total hexosamine content was estimated by the method of Cessi and Piliego (64).

Hexosamine analysis was also performed by the gas chromatographic procedure of Stimson (114). Aliquots of protein were hydrolysed as above and hexosamine hydrochlorides trimethylsilylated using bis (trimethylsilyl) trifluoroacetamide (Sigma (London) Chemical Co., Ltd.,) in dimethylacetamide (B.D.H.). Reference standards were prepared by the derivatizaton of D-glucosamine hydrochloride and D-galactosamine hydrochloride (Sigma (London) Chemical Co., Ltd.). Phenanthrene (B.D.H.) was included as an internal standard.

c). Estimation of Neutral Sugars

Neutral sugar was estimated colorimetrically by the anthrone procedure (68) after resin hydrolysis of the protein as described in Section A.

d) Estimation of Sialic Acids.

The thiobarbituric acid procedure of Warren (115) was used to assay sialic acids in the S - carboxymethyl microfibrillar preparation.

This/

This method involves the oxidation of sialic acids with periodate in strong acid solution, and the reaction of the oxidation product with 2 - thiobarbituric acid to yield a red chromophore which is extracted from the reaction mixture with cyclohexanone. As the assay measures only free sialic acids it is necessary that the protein be heated at 80°C for 1 hr in 0.1N H_2SO_4, a procedure known to release bound sialic acids without degradation (116,117).

An aliquot of the protein sufficiently large to allow the detection of 1 residue of sialic acid per 1000 amino acid residues was hydrolysed in H_2SO_4 and subjected to the assay procedure.

A solution of N - acetylneuraminic acid (Sigma (London) Chemical Co., Ltd.,) of known concentration was used to prepare a reference curve.

e). Amino end-group Determination

The N - terminal profile of the S - carboxymethyl microfibrillar component was determined by the 2 - chloro - 3,5 - dinitropyridine method, using the same experimental technique as for elastin, with the exception that 10 mg rather than 100 mg aliquots of the protein were subjected to the procedure.

Analytical Ultracentrifugation

The molecular weight of the S-carboxymethyl microfibrillar component was determined by meniscus depletion sedimentation equilibrium analysis (118). The instrument used was a Spinco Model E analytical ultracentrifuge (Beckman).

Suitable aliquots of the preparation were dissolved in 5M guanidine hydrochloride (62) - 0.1M Tris (pH 7.4) and dialysed exhaustively at 4°C against several changes of the same buffer. The density of the buffer solution was determined by picnometry.

The conditions for centrifugation were:

Temperature	-	20.4°C
Speed	-	39460 rpm
Cell	-	12 mm, with double sector centrepiece (two 2.5° sectors)
Optical system	-	Rayleigh interference optics

Fringe displacements were measured by means of a travelling microscope (Projectorscope, PG Ltd.), readings being taken at 200 micron intervals, commencing at the meniscus, along the X - scale, until a deflection of more than 10 microns occurred on the Y - scale between consecutive readings, which were then made at 100 micron intervals along the X - scale.

The partial specific volume of the protein was calculated from

its/

its amino acid composition.

The programme of Yphantis and Roark (119) was utilised in the computation of molecular weight values from experimental data.

RESULTS

The microfibrillar component was isolated from bovine ligamentum nuchae as the S - carboxymethyl derivative with a yield of 0.5% relative to the defatted, dry ligament.

CHEMICAL ANALYSES

a). Amino Acid Analysis

Table 21 reports the amino acid composition of the S - carboxymethyl microfibrillar component as isolated from mature bovine ligamentum nuchae (column a). Columns b and c of the same table present the two sets of compositional data as determined by Ross and Bornstein (22, 110) for similar preparations isolated from foetal bovine ligamentum nuchae. The concentrations of S - carboxymethyl cysteine, S - β - aminoethyl cysteine, cysteine and cysteic acid are totalled under the heading half-cystine.

Table 21 The Amino Acid composition of the Microfibrillar Component. (Values are expressed as residues/ 1000 Amino Acid residues and have been corrected for hydrolytic losses)

	a	b (22)	c (110)
Hydroxyproline	0.0	n.d.	
Aspartic Acid	107.6	114.0	127.0
Threonine	55.2	55.1	65.0
Serine	63.8	58.9	77.5
Glutamic Acid	106.1	111.0	136.0
Proline	56.2	70.4	60.5
Glycine	74.4	120.0	106.0
Alanine	61.7	58.9	63.0
½ Cystine	31.7	60.3	24.8
Valine	51.3	54.1	60.5
Methionine	19.8	15.8	13.5
Isoleucine	40.9	45.2	51.6
Leucine	86.3	57.2	74.2
Tyrosine	32.4	30.0	33.7
Phenylalanine	38.9	32.1	39.3
Lysine	84.7	36.9	39.3
Histidine	15.0	14.2	10.2
Arginine	68.9	45.2	54.0
Tryptophan	4.9	n.d.	24.8

b) Hexosamine, Hexose and Sialic Acid Analyses

Neither colorimetric analysis by the procedure of Cessi and Piliego nor gas chromatographic analysis revealed the presence of hexosamine in the S - carboxymethyl microfibrillar preparation.

Neutral hexose was not detected in the preparation upon analysis of resin hydrolysates by the anthrone method.

Sialic acids were absent from the preparation as indicated by the thiobarbituric acid assay procedure.

c). Amino End-group Determination

One amino acid only, namely glycine, was detected as an N - terminal residue in the S - carboxymethyl microfibrillar component by the chlorodinitropyridine method of end-group analysis. N - terminal glycine was present at a concentration of 65.9 moles per 10^6g of protein after correction for regeneration losses (70). This represents a minimum value as no allowance has been made for the loss of dinitropyridyl protein or amino acid during the various extraction stages of the procedure, and corresponds to a molecular weight of 15,170 daltons.

Analytical Ultracentrifugation

The molecular weight data derived from the analytical ultracentrifugation of two solutions of the S - carboxymethyl microfibrillar component in 5M guanidinium hydrochloride - 0.1M Tris (pH 7.4) are presented in Table 22 below.

Table 22. Molecular weight values for the S - carboxymethyl microfibrillar component.

	Solution I (~ 0.7 mg/ml)	Solution II (~ 0.3 mg/ml)
M - W	15 647 ± 239	14040 ± 156
M - z	19161 ± 94	15708 ± 136
M - z + 1	30361 ± 994	25699 ± 712
M - Y2	12167 ± 328	13011 ± 297
M - Y3	12768 ± 45	11431 ± 45
M - Y5	11979 ± 425	13639 ± 436
M - Y8	12746 ± 39	11275 ± 92

Concentrations were estimated by fringe counts. The concentration of solution II was the minimum for which it was possible to obtain sufficient fringe displacement measurements.

The M - Y values in Table 22 represent ideal molecular weight values to which various correction factors have been applied. The

M - Y8/

M - Y8 value supplies the most realistic estimate of molecular weight, having been corrected for the effects of concentration and non-ideality in the system.

DISCUSSION

In this study the microfibrillar component, present as a layer of tubular microfibrils surrounding the core of elastic fibres (105 - 112), was isolated from mature bovine ligamentum nuchae in a manner similar to that described by Ross and Bornstein for foetal ligament (22). The two isolated procedures did, however, differ with respect to the sequence of the various purification stages. In the present investigation the microfibrillar component was isolated from ligamentum nuchae previously extracted with 5M guanidine, and contaminating collagen then removed by the use of collagenase purified by affinity chromatography. Ross and Bornstein, on the other hand, isolated the microfibrillar component from guanidine extracted, collagenase treated ligamentum nuchae. In both cases the component was solubilised by the reduction of disulphide bonds, reformation of these bonds being prevented by the carboxymethylation of the thiol groups.

In term foetal ligamentum nuchae the microfibrils were found to represent approximately 5 - 10% of the dry weight of the elastic fibres (22), in marked contrast to the value of 0.7% reported in the present investigation for mature ligament (assuming the elastic fibres to be present at a concentration of approximately 70% of the dry weight of defatted ligament (1)). This discrepancy may reflect the higher content of the microfibrillar component in foetal ligament, the microfibrils being the first structures to appear in the

morphogenesis/

morphogenesis of elastic fibres (106 - 109, 111, 112). However, owing to the rather low molecular weight of the S - carboxymethyl microfibrillar component (approximately 12 000 daltons) material may have been lost during the periods of dialysis involved in the present isolation scheme, since standard, rather than acetylated, Visking tubing was used (120).

The amino acid compositions of the two preparations (Table 21, columns a and b) are markedly different, that of Ross and Bornstein Table 21, column b) exhibiting a much higher content of glycine, proline, and half-cystine and a lower content of lysine and arginine. In a later investigation, Ross and Bornstein published a rather different amino acid composition for the material (Table 21, column c) showing a reduced content of proline, glycine and half-cystine (110). Although rather more similar to the amino acid composition reported in the present study there are substantial differences, particularly with respect to lysine and glycine. The preparations also differ with regard to their carbohydrate contents. Neither hexose nor hexosamine were detected in the present case, while the preparation isolated by Ross and Bornstein contained between 4-7% by weight of hexose and approximately 0.7% hexosamine (110).

The purity of the S - carboxymethyl microfibrillar preparation isolated in this study is evident from the presence of only one

amino/

amino acid as N - terminal residue, namely glycine. The concentration of N - terminal glycine, corresponding to a molecular weight of approximately 15 000 daltons, is in good agreement with the results of analytical ultracentrifugation which indicated a value of between 14 000 and 15600 daltons as the weight average molecular weight of the alkylated microfibrillar component. However, it must be mentioned that when corrections were made for the effects of non-ideality and concentration a rather lower estimate of molecular weight was obtained - approximately 12 000 daltons. On the other hand the molecular weight as assessed from amino end group determinations represents a maximum value, no allowance having been made for the possible loss of material during the analysis.

BIBLIOGRAPHY

1. John R. & Thomas, J. (1971). Int., J. Biochem., 2, 529

2. Gotte, L., Stern, P., Elsden, D.F. & Partridge, S.M. (1963). Biochem. J., 87, 344

3. Partridge, S.M., Elsden, D.F. & Thomas, J. (1963). Nature (London), 197, 1297.

4. Thomas, J., Elsden, D.F. & Partridge, S.M. (1963). Nature (London), 200, 651.

5. Anwar, R.A. & Oda, G. (1966) Nature (London), 210, 1254

6. Anwar, R.A. (1966). Can. J. Biochem. Physiol, 44, 725

7. Ledvina, M. & Bartoš, F. (1970). Hoppe-Seyler's Zeitschrift Für physiologische Chemie, 351, 661

8. Franzblau, C., Sinex, F.M. & Faris, B. (1965). Nature (London) 205, 802

9. Franzblau, C., Sinex, F.M., Faris, B. & Lampidis, R. (1965). Biochem. Biophys. Res. Comm., 21, 575

10. La Bella, F.S. & Vivian, S. (1967). Biochim. Biophys. Acta, 133, 189

11. La Bella, F.S., Keeley, F., Vivian, S. & Thornhill, D. (1967) Biochem. Biophys. Res. Comm., 26 , 748.

12. Lent, R.W., Smith, B., Salcedo, L.L., Faris, B. & Franzblau, C. (1969), Biochem., 8, 2837

13. Adair, G.S., Davis, H.F. & Partridge, S.M. (1951). Nature (London), 167, 605

14. Bendall, J.R. (1955). Biochem. J., 61, 31

15. Partridge, S.M., Davis, H.F. & Adair, G.S. (1955). Biochem. J., 61, 11

16./

16. Partridge, S.M., Davis, H.F. (1955). Biochem. J., 61, 21

17. Lansing, A.I., Rosenthal, T.B., Alex, M. & Dempsey, E.W. (1952) Anat. Rec., 114, 555

18. Serafini-Fracassini, A. & Smith, J.W. (1974). The Structure and Biochemistry of Cartilage, p.7, Churchill Livingstone, Edinburgh and London

19. Hospelhorn, V.D. & FitzPatrick, M.J. (1961). Biochem. Biophys. Res. Comm., 6, 191

20. Miller, E.J. & Fullmer, H.M. (1966). J. Exp. Med., 123, 1097

21. Steven, F.S. & Jackson, D.S. (1968). Biochim. Biophys. Acta, 168, 334

22. Ross, R. & Bornstein, P. (1969). J. Cell. Biol., 40, 366

23. Grant, M.E., Steven, F.S. & Jackson, D.S. (1971), Biochem. J., 121, 197

24. Bailey, A.J. & Etherington, D.J. (1970). Biochim. Biophys. Acta, 214, 238

25. Robert, B. & Robert, L. (1969). Eur. J. Biochem., 11, 62

26. Lansing, A.I., Roberts, E., Ramasarma, G.B., Rosenthal, T.B. & Alex, M. (1951). Proc. Soc. Exp. Biol. Med., 76, 714

27. Hall, D.A. (1955). Biochem. J., 59, 459

28. Gotte, L., Serafinin-Fracassini, A. & Moret, V. (1963). J. Atherscl. Res., 3, 244

29. Moret, V., Serafini-Fracassini, A. & Gotte, L. (1964). J. Atherscl. Res., 4 184

30./

30. FitzPatrick, M. & Hospelhorn, V.D. (1965). Am. Heart J., 69, 211

31. Gotte, L., Meneghelli, V. & Castellani, A. (1965), Structure and Function of Connective and Skeletal Tissue, p.93. Ed. Fitton-Jackson, S., Partridge, S.M., Harkness, R.D., & Tristram, G.R. London: Butterworths

32. Loeven, W.A. (1965). Acta physiol. pharmacol. neerlandica, 13, 278

33. La Bella, F.S., Vivian, S. & Thornhill, D.P. (1966). J. Geront., 21, 550

34. Serafini-Fracassini, A. & Tristram, G.R. (1966). Proc. Royal Soc. Edinburgh, 69B, 334

35. Hall, D.A. (1968). Exp. Geront., 3, 77

36. Weissman, N., Shields, G.S. & Carnes, W.H. (1963). J. Biol. Chem., 238, 3115

37. Smith, D.W., Weissman, N & Carnes, W.H. (1968). Biochem. Biophys. Res. Comm., 31, 309

38. Sandberg, L.B., Hackett, T.N. Jr. & Carnes, W.H. (1969). Biochim. Biophys. Acta, 181, 201

39. Smith. D.W. & Carnes, W.H. (1973). J. Biol. Chem., 248 8157

40. Siegel, R.C., Pinnell, S.R. & Martin, G.R. (1970). Biochem., 9, 4486

41. Sandberg, L.B., Weissman, N. & Gray, W.R. (1971). Biochem., 10,52

42./

42. Sandberg , L.B., Zeikus, R.D. & Coltrain, I.M. (1971). Biochim. Biophys. Acta, 236, 542

43. Sandberg , L.B., Bruenger, E. & Cleary, E.G. (1975). Analyt. Biochem., 64, 249

44. Sandberg, L.B., Weissman, N. & Smith D.W. (1969). Biochem., 8, 2940

45. Keller, S., Levi, M.M. & Mandl, I. (1969). Arch. Biochem. Biophys., 13,565

46. Shimada, W., Bowman, A., Davis, N.R. & Anwar, R.A. (1969). Biochem. Biophys. Res. Comm., 37, 191

47. Thomas. J. (1971). Int. J. Biochem., 2, 644

48. Robert, L. & Poullain, N. (1966). Arch. maladies du coeur, des vaisseaux et du sang, Suppl., 8, 121

49. Gotte, L. & Serafini-Fracassini, A. (1963). J. Atherscl. Res., 3, 247

50. Gotte, L., Giro, M.G., Volpin, D. & Horne, R.W. (1974), J. Ultrastruct. Res., 46, 23

51. Cox, R.W. & O'Dell, B.L. (1966). J. Royal Microsp. Soc., 85,401

52. Gotte, L., Mammi, M. & Pezzin, G. (1968). Symp. Fibrous. Proteins, p.236. Ed. Crewther, W.G. London: Butterworths.

53. Mammi, M., Gotte, L. & Pezzin, G. (1970). Nature (London), 225, 380

54. Treloar, L.R.G. (1958). The Physics of Rubber Elasticity. Oxford: Clarendon Press

55./

55. Hoeve, C.A.J. & Flory, P.J. (1958). Jl Am. Chem. Soc., 80, 6523

56. Weis-Fogh, T. & Andersen, S.O. (1970). Nature (London), 227, 718

57. Hoeve, C.A.J. & Flory, P.J. (1974). Biopol., 13, 677

58. Yoshida, E. & Noda, H. (1965), Biochim. Biophys, Acta, 105, 562

59. Gotte, L., Pezzin, G. & Stella, G.D. (1966) in Biochemie et Physiologie du Tissu Conjonctif, pp. 145 - 154. Ed. Comte, P. Lyon: Ormeco

60. Mistrali, F., Volpin, D., Garibeldo, G.B. & Ciferri, A.(1971). J. Phys. Chem., 75, 142

61. Moore, A.T. & Stein, W.H. (1948). J. Biol. Chem, 176, 367

62. Nozaki, Y. (1972). Methods Enzymol., 26, 43

63. Blumenkrantz, N. & Asboe-Hansen, G. (1973). Analyt. Biochem., 55, 288

64. Cessi, C. & Piliego, F. (1960). Biochem. J., 77, 508

65. Anastassiadis, P.A. & Common, R.H. (1958). Can. J. Biochem., 36, 413

66. Luscombe, M. & Phelps, C.F. (1967). Biochem. J. 102, 110

67. Bhatti, T., Chambers, R.E. & Clamp, J.R. (1970). Biochim. Biophys. Acta, 222, 339

68. Scott, T.A. & Melvin, E.H. (1953). Analit. Chem, 25, 1656

69. Signor, A., Previero, A. & Terbojevich, M. (1965). Nature (London), 205, 596

70./

70. Signor, A. & Biondi, L. (1964). Ric. Sci., 4, 165

71. Stark, G.R. & Smyth, D.G. (1963). J. Biol. Chem., 238, 214

72. Ward. D.N., Coffey, J.A., Ray, D.B. & Lamkin, W.M. (1966) Analyt. Biochem., 14 243

73. Kang, A.H., Bornstein, P. & Piez, K.A. (1967). Biochem., 6, 788

74. Mellema, J.E., van Bruggen, E.F.S. & Gruber, M. (1968). J. Mol. Biol., 31, 75

75. Whiting, A.H., Sykes, B.C. & Partridge, S.M. (1974). Biochem. J., 141, 573

76. Valentine, R.C. (1958). Proc. 4th Int. Conf. Electron Microscopy, 1, 577. Berline: Springer Verlag

77. Smith, D.W., Brown, D.M. & Carnes, W.H. (1972). J. Biol. Chem., 247, 2427

78. Sanger, F. (1945). Biochem. J., 39, 507

79. Porter, R.R. & Sanger, F. (1948). Biochem. J., 42, 287.

80. Narita, K. p.28 in Protein Sequence Determination, Ed. Needleman, S.B. 1970, London: Chapman & Hall Ltd.

81. Taylor, K.B. (1964) Nature 202 , 1217

82. Sheehan, J.C. & Yange, D.D.H. (1958). J. Am. Chem. Soc. 80, 1154

83. Partridge, S.M. Elsden, D.F., Thomas J. Dorfman, A., Telser, A. & Pei-Lee, Ho (1964). Biochem. J., 93, 30C

84. Partridge, S.M., Thomas J. & Elsden, D.F. (1964). Structure and Function of Connective and Skeletal Tissue, p.83. Ed. Fitton-Jackson, S., Partridge, S.M., Harkness, R.D & Tristram, G.R. London: Butterworths.

85./

85. Miller, E.J., Pinnell, S.R. Martin, G.R. & Schiffman, E. (1967). Biochem. Biophys. Res. Comm., 26, 132

86. Lent, R. & Franzblau, C. (1967). Biochem. Biophys. Res. Comm., 26, 43

87. Anwar, R.A. & Oda. G. (1966). J. Biol. Chem., 241, 4638

88. Piez, K.A. (1968). Ann. Rev. Biochem., 37, 547

89. Franzblau, C., Faris, B. & Papaioannou, R. (1969) Biochem., 8, 2833

90. Partridge, S.M. (1969). Gerontologia, 15, 85

91. Franzblau, C. (1971). Comprehensive Biochemistry, vol.26C p.659. Ed. Florkin, M. & Stotz, E.H. Amsterdam: Elsevier

92. Ramachandran, G.N. p.127 in International Review of Connective Tissue Research, Vol. 1, 1963. Ed. Hall, D.A.

93. Schmitt, O.F. p.1129 in Medical Physics, Vol.2. Ed. Glasser, O. Chicago: The Year Book Publishers.

94. Davril, M. & Nan, Kia-Ki. (1974). Febs. Letters, 43, 331

95. Foster, J.A., Bruenger, E., Gray W.R. & Sandberg L.B. (1973) J. Biol. Chem., 248, 2876

96. Foster, J.A., Rubin, L., Kagan, H.M., Franzblau, C., Bruenger, E. & Sandberg, L.B. (1974). J. Biol. Chem., 249, 6191

97. Torchia, D.A. & Piez, K. (1973). J. Molec. Biol. 76, 419

98. Frushour. B.G. & Koenig, J.L. (1975). Biopolymers, 14, 379

99. Urry, D.W. (1971). Proc Natn, Acad. Sci. U.S.A., 68, 810

100./

100. Abatangelo, G. Daga-Gordini, D. & Tamburro, A.M. (1973). Int. J. Pept. Prot. Res., 5,63

101. Urry, D.W., Cunningham, W.D. & Ohnishi, T. (1974). Biochem., 13, 609

102. Cox, B.A., Starcher, B.C. & Urry, D.W. (1974). J. Biol. Chem., 249, 997

103. Cox, B.A., Starcher, B.C. & Urry, D.W. (1973). Biochim. Biophys. Acta., 317, 209

104. D.W. Urry, Long, M.M. Cox, B.A., Ohnishi, T., Mitchell, L.W. & Jacobs, M. (1974). Biochim. Biophys. Acta., 371, 597

105. Usuku, G. (1958). Kumanoto Med. J., 11, 84

106. Fahrenbach, W.H., Sandberg. L.B. & Cleary, E.G. (1966). Anat. Rec., 155, 563

107. Greenlee, T.K., Ross R. & Hartman, J.L. (1966). J. Cell Biol., 30,59

108. Ross, R. (1968). Treatise on Collagen. Ed. Gould., B.S. London: Academic Press

109. Karrer, H.E. (1960). J. Ultrastruct. Res., 4, 420

110. Ross. R. & Bornstein, P. p.641 in Chem. and Mol. Biol. of the Intercellular matrix. Vol.1. Ed. Balazs, E.A. London: Academic Press

111. Karrer, H.E. (1961). J. Ultrastruct. Res., 5, 1

112. Low. F.N. (1962). Anat. Rec., 142, 131

113./

113. Opienska-Blauth, J., Charezinski, M. & Berbec, H. (1963). Analyt. Biochem., 6, 69

114. Stimson, W.H. (1971). Febs. Letters, 13, 17

115. Warren, L. (1959). J. Biol. Chem., 234, 1971

116. Svennerholm, L. (1958). Acta Chem. Scand. 12, 547

117. Warren, L. (1959). J. Clin. Invest., 38, 755

118. Chervenka, C.H. (1970). Analyt. Biochem., 34, 24

119. Yphantis., D.A. & Roark, D.E. (1972). Biochem, 11, 2925.

120. Craig. L.C. & Konigsberg, W. (1961). J. Phys. Chem., 65, 166